The Role of Hippocampal Dendrites in Evoked Responses and After-discharges.

By

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The hippocampus (Ammon's horn) is a favorable site for the
investigation of cortical properties (RENSHAW, FORBES and Mo-
RISON 1940) and the role which dendrites play in evoked potentials.
Its anatomy has received careful study by CAJAL (1911, 1955),
LORENTE DE NÓ (1934) and many other histologists. The lamina-
tion of the hippocampal cells is simple and uniform. Surgical
approach to the hippocampus may be made without interference
to its blood supply, and surgical section, or removal of adjacent
structures, can be undertaken to simplify the recording conditions.
The present study was designed to take advantage of these fea-
tures, displayed schematically in Fig. 1. The results seem to show
that, following single volleys to the dorsal fornix, the distal parts
of the pyramidal dendrites are not actively depolarized but mas-
sive depolarization of the dendritic layer occurs after repetitive
stimulation. This is apparently associated with the mechanism
of seizure discharges to which the hippocampus is particularly
susceptible (GIBBS and GIBBS 1936; JUNG 1949; KAADA 1951;
GREEN and SHIMAMOTO 1953).

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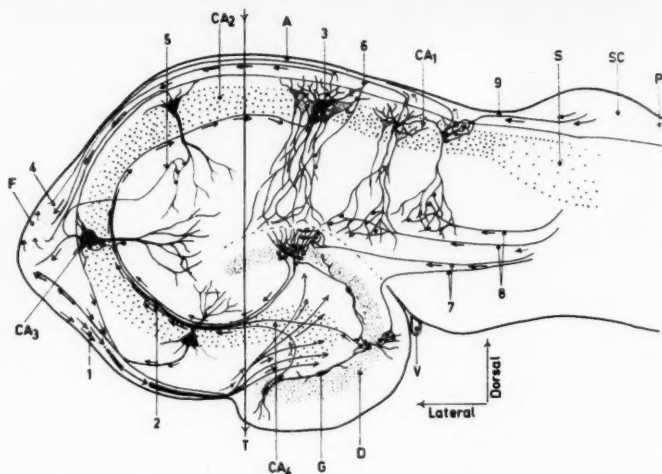Roma, Italy.

Fig. 1. Schema of Dorsal Hippocampus. The vertical line (T) indicates the usual recording track. A — Alveus. D indicates gyrus dentatus. F — Fimbria. The pathway considered here begins at (1) where afferents (derived from the septum or hippocampal commissure) enter the hilus of the gyrus dentatus, relay in the vicinity of the granule cells, the axons of which (2) gain relay on the hippocampal pyramids (3). The axons of the pyramidal cells enter the fimbria (F) and (4). Long axon collaterals arise in area CA 3 (5) and end among the pyramidal dendrites. Short collaterals (6) are also present. The temporo-ammonic tract (7, 8) arising from cells in the subiculum (S) ends among the apical dendrites of both the pyramidal and granule cells. Afferents supposedly from the spheno-cornual bundle (SC) are indicated at (9) and are presumably accompanied by some fibres from the psalterium (P). At V is indicated the site of entrance of the septal vessels which follow the cleft between dentate and pyramidal cells. G represents Golgi type II cells of the dentate gyrus which may be found in this position.

Previously (GREEN and MACHNE 1955; GREEN and ADEY 1956) evidence was advanced that stimulation of the dorsal fornix evoked a post synaptic response in the hippocampus. This response had a latency of 4—5 msec, was potentiated by strychnine, inhibited by anoxaemia or locally applied nembutal and was not abolished by section of the fibres of the alveus around the responding region (GREEN and ADEY 1956). Furthermore, the changes of polarity of the response recorded with concentric electrodes with depth and the results of interrupting various afferent paths indicated that the afferent fibres for this response entered the hippocampus near the junction of the fimbria and gyrus dentatus. It seemed reasonable to suppose, therefore, that these impulses entered the gyrus dentatus, relayed there and were relayed by

the granule cells to the pyramidal cells along this well-known intra-hippocampal pathway. On the basis of what is known about the structure of the hippocampus, the only reasonable alternative seemed to be that excitation of the efferent fibres of the fornix antidromically activated axon collaterals, which in turn orthodromically excited adjacent neurones. However, GREEN and ARDUINI (1954) showed that the theta rhythm of the rabbit hippocampus was maintained through the dorsal fornix so that there is good reason to think that at least some afferents are conveyed in this manner, and that antidromic activation is, therefore, unlikely to be the complete explanation. The results of the present study tend to confirm the pathway GREEN and ADEY (1956) proposed, which is somewhat similar to a pathway in the ventral psalterium suggested by CRAGG and HAMLYN (1957) in a recent paper.

It was decided, therefore, to study these evoked potentials extracellularly, using fine electrodes inserted into area CA_2 (H_2) of the dorsal hippocampus under direct vision. Here, the alveus and layer of basal dendrites extend 0.1—0.2 mm from the surface, the cell layer extends from 0.2—0.4 mm, the thick unbranched portions of the apical dendrites down to about 0.6 mm and the branched portion from about 0.8 to 1.35 mm. The axons of the granule cells of the gyrus dentatus synapse about the cell bodies and unbranched portions of the apical dendrites between about 0.3 mm and 0.6 mm from the surface. Although the granule cell axons are most evident in areas CA_3 and CA_4 their terminations and their branches can be followed as far as CA_1.

The terminals of the temporo-ammonic tracts synapse chiefly in the region of the branched portions of the apical dendrites (CAJAL 1955), but these synapses may, presumably, be disregarded in the present study since the cells from which the temporo-ammonic axons arise are removed when the entorhinal cortex is removed.

Materials and methods.

Cats have been used, with a few confirmatory experiments on rabbits. The majority of the experiments were carried out as follows: under general anaesthesia the cerebral cortex was exposed, and its surface coagulated with silver nitrate. The lateral ventricles were opened by removal of the cerebral cortex. As fas as possible, the entorhinal cortex was ablated: in many cases completely. Thus, all but the frontal cortex and the rostral extremity of the temporal lobes was

removed. After careful block of all the peripheral nerves to the scalp and pressure points with one per cent procaine and application of procaine to all areas of incision (tracheotomy and venous cannula site), ether anaesthesia was discontinued and immobilization secured with minimal doses of tubo-curarine. The preparation was, therefore, essentially a decorticate animal with exposed pain receptors additionally blocked by local anaesthesia.

Electrodes were placed in position and the effects of ether allowed to wear off for about one hour before recording was commenced. The operation permits the investigator to keep the hippocampus exposed under warm mineral oil for long periods with every indication of its viability. Subsequent histological studies have shown excellent preservation. The fornix and psalterium may be sectioned and other surgical procedures carried out. To cut the fornix or psalterium, a fine hypodermic needle was attached to the suction apparatus and the sharp edge used as a knife. The suction keeps the field clear and helps to avoid injury to the tela choroidea. Recording electrodes were inserted at Horsley Clarke planes A_4 to A_5, about 1 1/2 to 2 mm medial to the fimbria. Coaxial stimulating electrodes were placed in the dorsal part of the fornix and septum pellucidum. To make cuts in the hippocampus small guarded knives were made with fragments of razor blade which were clamped in haemostats and adjusted under a micro-projector until the fragment projected the distance of the desired depth of cut.

Ringer-filled micro-pipettes of 3—10 M Ω resistance or metal electrodes with tips of about one micron but somewhat larger uninsulated area were used. The potentials were recorded between them and large distant reference electrodes. In addition, surface records were taken with silver ball electrodes. For D. C. recording, Ringer-filled pipettes and silver-silver chloride leads were used, with chlorided silver reference electrodes wrapped in saline soaked cotton. For vector recording, enameled nichrome wires (no. 36) were sheared at the tip and embedded in acrylic resin to the desired separations. The tips described right angle triangles with the common electrode at the apex of the right angle (see p. 102 and Fig. 11).

Results.

Stimulating the dorsal fornix with single shocks and recording with a small 'active' electrode against a distant reference electrode at successive depths of area CA_2 and the underlying dentate gyrus (see Fig. 1) a series of records may be obtained such as those shown in Fig. 2. In this illustration, which is a key figure for the development of our thesis, sample records from a typical series are shown at various depths of a single electrode track. The records were taken at intervals of 0.1 mm with 4 or 5 sweeps super-
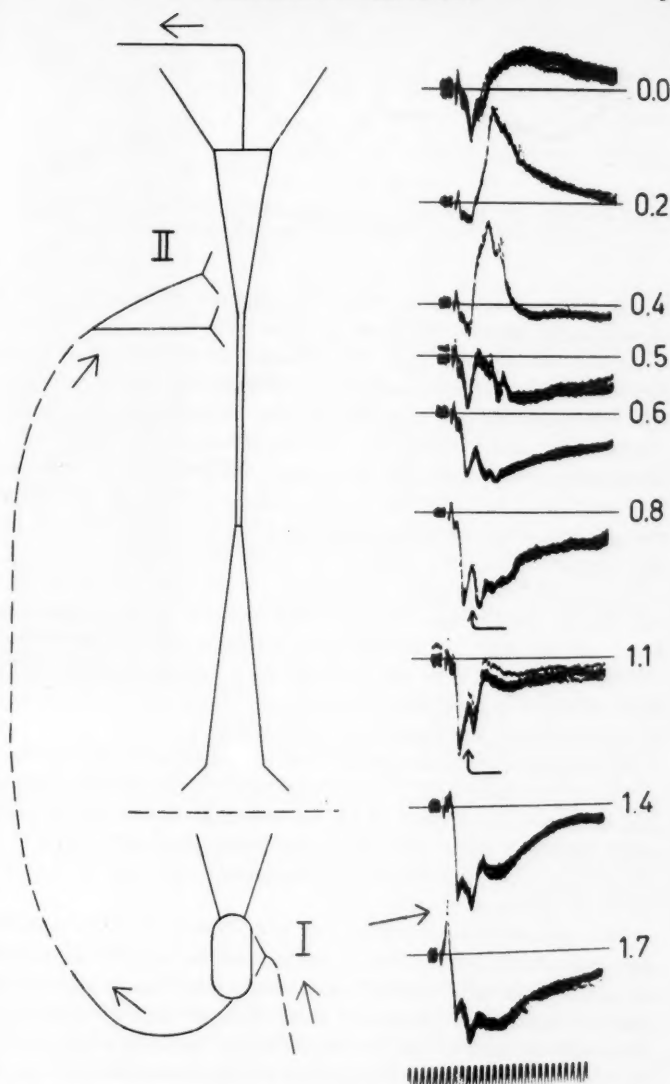
Fig. 2. Focal records (superimposed sweeps) selected from a series at 0.1 mm steps. Depths in mm. Time line 500 c. p. s. In this and all subsequent records negativity is represented upward. The electrode path is shown in Fig. 4. Diagram represents the approximate positions of the various parts of the pyramidal cell layer (above) and granule cell layer, below.
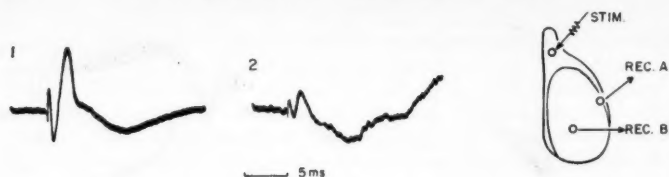
Fig. 3. Stimulation at point indicated on dorsal fornix. Surface records at threshold for post-synaptic response 1, at A, close to fimbria; 2, at B.

imposed at each level. Two subsequent figures (4 and 5) are based on the series from which these samples were taken.

On the left of the figure the pathway we are investigating is illustrated schematically in such a way that the sweeps lie opposite corresponding cell components. For reasons given previously (GREEN and ADEY 1956) and further developed below, it is believed that the afferents relay first onto the granule cells (synapse I of the figure) and that the granule cells relay a second time near the cell bodies of the pyramidal cells (synapse II near the top of the picture). This path is shown in more detail in Fig. 1.

The earliest obtainable component of the evoked potential is not always easily seen. It is a sharp diphasic potential with latency of less than 1 msec. While not clearly seen in Fig. 2 it is obvious in Fig. 3 (1) or Fig. 7 (B, 2). It is believed to represent a fiber response in the fimbria and alveus. If it is partly antidromic it fails to invade the pyramidal cell somata.

The second deflexion is also small and sharp. It has a latency of about 1.5 msec and may be observed in the lowest trace of Fig. 2 where it is indicated by an arrow. It appears as a sharp early negative deflexion. The maximum amplitude occurs at about 2 msec. This response is interpreted to be due to the synchronous firing of granule cells.

The main deflexion has a latency of 4—5 msec. It has a negative sign only at the level of the pyramidal cell bodies. The amplitude at this level is quite variable and usually less than is seen in Fig. 2 (second and third traces at 0.2 and 0.4 mm depths). Above and below the layer of cell bodies the deflexion normally has a positive sign, suggesting that synapse II is being activated close to the cells. It also indicates that propagation is blocked in the dendritic layer, a point to be further elaborated below.

Several features are puzzling on causal inspection of records such as those of Fig. 2. The questions may be raised: 1) Are distant
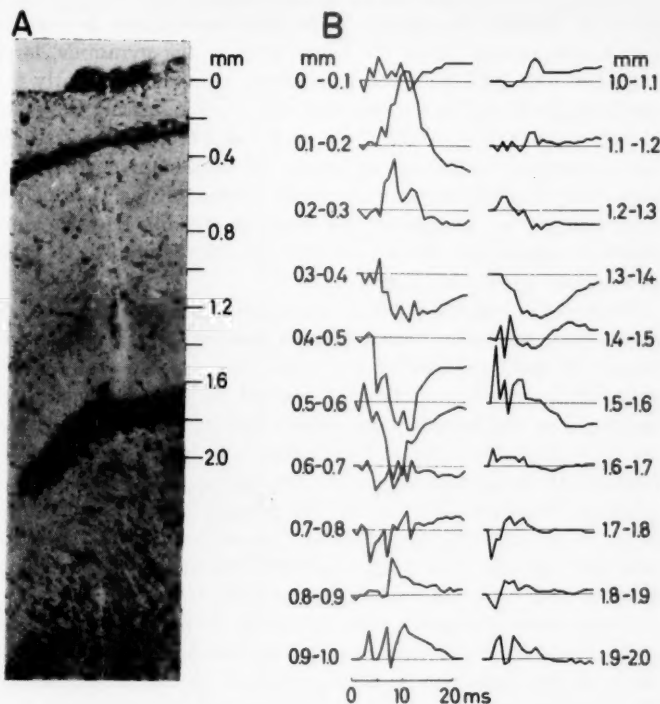
Fig. 4. *A*. Electrode track along which the original records were obtained, which are shown in Fig. 2 and from which the curves in B and in Fig. 5 are constructed. The track was heavily marked by moving the electrode far down and up several times. Nissl stain. *B*. Family of curves representing gradients of response amplitude at successive depth in hippocampus along the electrode track shown in A. The curves are constructed as the difference between every two records at 0.1 mm steps. Upward deflection represents relative negativity at the lower depth. Samples of the series of original records are shown in Fig. 2.

current generators responsible for the shapes of the potentials? 2) Does the almost exclusively positive sign of the dendritic layer responses truly indicate blocked propagation? 3) May latency differences be detected at different levels? 4) What is the significance of the rather variable small notch in the positive wave (Fig. 2 traces at 0.8 and 1.1 at arrow)?

To exclude the effects of distant current generators and to emphasise significant detail, voltage differences were measured in successive records at intervals of 0.1 mm. Gradient curves (equiv-

alent to bipolar recordings) were then constructed as seen in Fig. 4. Above and below the soma layer of the pyramids, large gradients with maxima at about 9 msec, are seen. Evidently the direction of current flow changes at this level indicating the point of maximum current inflow. Between 0.7 and 0.9 mm the gradient again reverses, indicating the region of maximum current out-flow. This is clearly seen also in the curves of Fig. 5, which is described below. Thereafter the gradient steadily declines until changes, apparently attributable to the first synaptic relay in the dentate gyrus, are seen.

Since there was an apparent blockage of somatofugal conduc-tion along the dendrites and this seemed to us to be a point of major interest, we decided to display the data in still another way which would amplify any gradual shift of latencies with depth. Thus amplitudes were plotted against distance, one curve for each time instant from the stimulus with intervals of 0.9 msec (Fig. 5). By inspection of such curve families, slow rates of pro-pagation should readily be seen as gradual shifts in position of negative peaks along the abscissa. The accuracy of the method was such that we should have detected changes of peak positions moving with rates between 1.0 m/sec and 15 mm/sec.

At 1.8 msec a negative peak is seen at the level of the granule cells. At 2.7 msec this peak has virtually disappeared. At 3.6 msec there is very little sign of any gradient along the track, but the 5.4 msec curve shows a negative peak at the pyramidal level which grows in subsequent curves. Except for a slight shift in position of the second peak between 9 and 16.2 msec, it does not propagate. It is noteworthy that a marked voltage gradient be-tween cell layer and dendrites persists in all the subsequent curves (up to 21.6 msec). The later curves at about 1.2 mm approach zero. This level may represent the point where outward current of the pyramids ceases and outward current of the granule cells be-gins. The relevance of these data to propagation will be discussed below.

The focal responses show some variation from one animal to another. They may also be profoundly affected by repetitive stim-ulation or by drugs. In contrast to the earlier records obtained with coaxial electrodes (GREEN and ADEY 1956) potentiation in the pyramidal layer was not as a rule very marked. At least as frequently the responses were seen to decline in amplitude. In addition they changed shape with repetitive stimulation, becom-
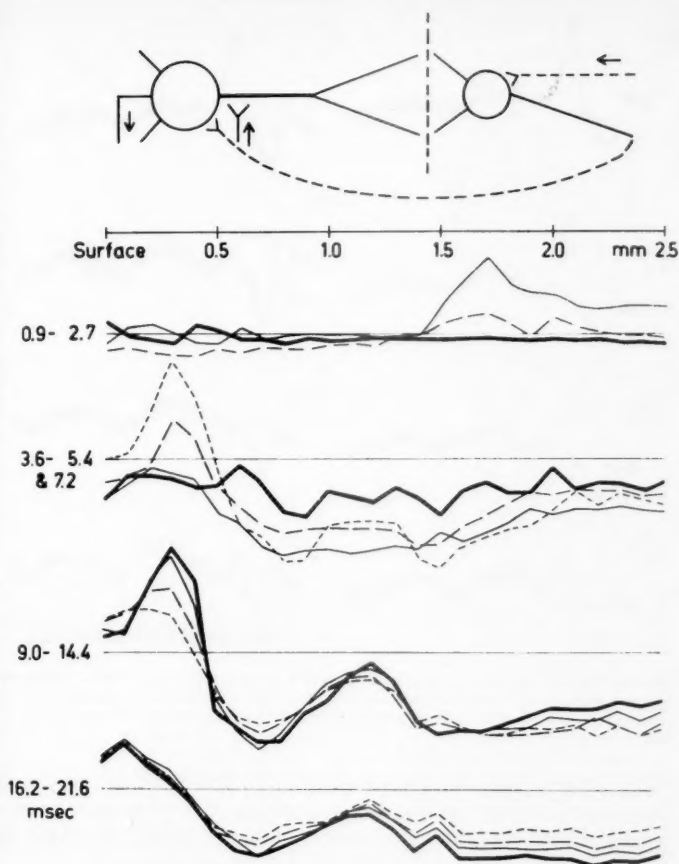
Fig. 5. Family of curves representing response amplitude versus depth at successive times. The curves are arranged in four groups: 0.9; 1.8; 2.7 msec in the top group, 3.6; 4.5; 5.4; 7.2 msec in the second group, 9.0; 10.8; 12.6; 14.4 msec in the third group and 16.2; 18.0; 19.8; 21.6 msec in the bottom group. In each group the heavy line represents the earliest time and then in order the thin solid line, the broken line and the dashed line.

ing more negative at the dendritic level (see below). If records were made too soon after the hippocampus had been exposed, extremely variable responses were seen often complicated by after discharges. Administration of nembutal tended to block the after discharges but also abolished the negative response at the
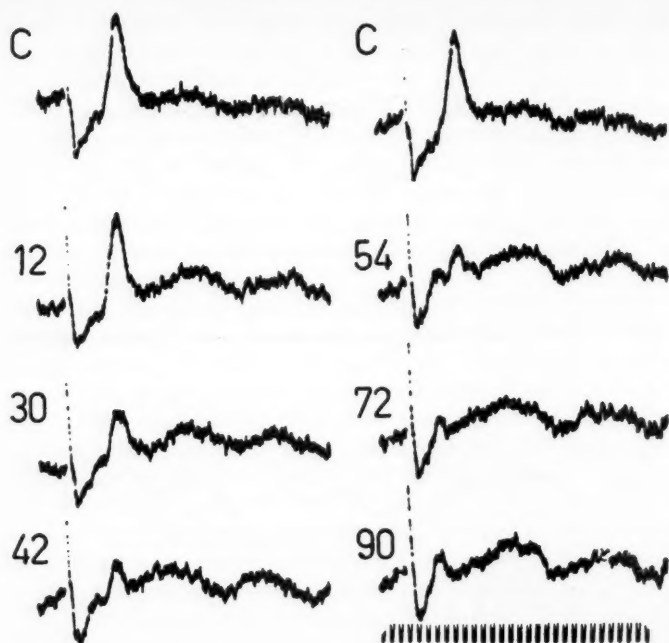
Fig. 6. Effect of Nembutal on response at level of pyramidal cell bodies. C, C. control records before drug. Subsequent sweeps at 12, 30, 42, 54, 72 and 90 seconds after intravenous injection of 10 mg Nembutal per kg body weight. Time 500 c. p. s.

level of the pyramidal cell bodies (Fig. 6). The responses may also be complicated by the simultaneous stimulation of more than one group of afferents. The response described here can be obtained by stimulation of the ipsilateral dorsal fornix or septum.

Stimulation of the contralateral dorsal fornix gives rise to a response of considerably longer latency and different wave-shape (Fig. 7). This may indicate that when the contralateral fornix is stimulated it excites the hippocampus of that side in turn affecting the ipsilateral hippocampus through the ventral commissure, along the path described by CRAGG and HAMLYN (1957). Frequently responses from the contralateral side are very complex and show multiple curves with high intensity stimulation. To ensure that the responses recorded did not necessarily pass first to the contralateral side, we cut the psalterium and obtained the results shown in Fig. 7. We also repeated earlier experiments of cutting the fibres of the alveus. Neither of these procedures abolished the response recorded from the surface of the hippo-
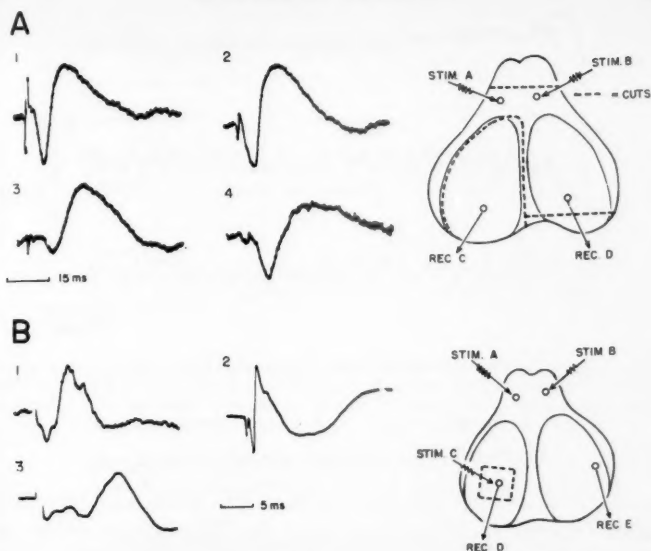
Fig. 7. Diagrams at right illustrate arrangement of electrodes. *First series.* Cuts as at dotted lines. Fornix and septum sectioned. Psalterium cut as indicated. Deep posterior cut on right, isolating caudal part of R. hippocampus. Cut 0.7 mm deep at alvear-fimbrial junction at left. (1) Single shock to B, record from D. (2) Single shock at A, record from C. (3) Single shock at B, record at C. (4) Single shock at A, record at D. The increased latency of the crossed responses suggests that they are due to impulses travelling from one side to the other through the rostral (uncut) end of the psalterium after relaying ipsilaterally. Record (2) indicates that alvear fibres are not essential for the response, but note loss of early sharp potential compared with 1. *Second series.* Effect of cutting alveus and other afferent connections. In 1 and 2, only the alvear fibres were cut. In 3 the cut was extended through the whole pyramidal layer. In 1 the stimulus was made at A and response recorded at D. The response lacks the early sharp biphasic potential. In 2, B was stimulated and the record obtained at E. The early fibre response is prominent because the recording electrode is close to the fimbria. In 3, the cuts were extended below the level of the pyramidal dendrites. Stimulation at C still evoked a response of long (and variable) latency at E, despite section of all normal connections.

campus but we did not explore the whole depth of the pyramidal layer in this way. Section of the psalterium or spheno-cornual bundle does not modify the surface response.

Experimental modification of hippocampal evoked potentials. As indicated above, a small notch (Fig. 2, arrows at 0.8 and 1.1 mm) is often seen near the apex of the large positive potential in the dendritic field. This notch is quite variable and sometimes absent. It is not possible to measure its latency precisely but its peak is
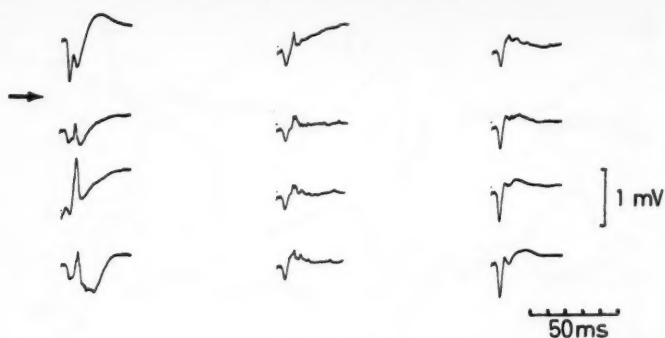
Fig. 8. Record at depth of 0.8 mm. The first trace is a control. At the arrow the dorsal fornix received a 1 second burst at 100 c. p. s. Subsequent records at intervals of 1 second. Note growth of 'notch' to negative potential and subsequent slow (and incomplete) recovery. For further details, see text.

about 10 msec after the shock artifact. The notch may be present after removal of all connexions of the hippocampus except the fornix. Following a brief burst of repetitive stimulation it becomes a clear cut negative peak (Fig. 8). When the fornix is stimulated repetitively at a rather low frequency (about 10/sec) the notch may appear where it was previously absent. Further stimulation (Fig. 9) leads to a complete inversion of the positive deflexion in the dendritic field. If stimulation is stopped just at the point of complete inversion, an after-discharge of potentials of the same general shape ensues. If, however, stimulation is continued beyond the point of maximum inversion a seizure discharge occurs before stimulation ceases. This curious inversion is also seen in Fig. 10. Somewhat similar effects have been noted by ADRIAN (1936) and many other authors in neocortex.

It may be seen in Fig. 9 that the progressive inversion of the potential is by no means a simple occurrence. No indication of any dimensional change or movements of the hippocampus were ever noted during these experiments. The inversion effect at the surface was seen by GREEN and ADEY (1956) using ball electrodes which could not have changed position. As Fig. 8 shows the inversion can occur very rapidly indeed. Thus, despite the very marked gradients that occur in the hippocampus, there is no reason to think that electrode movement could explain these changes. Even if electrode movement did occur we cannot conceive

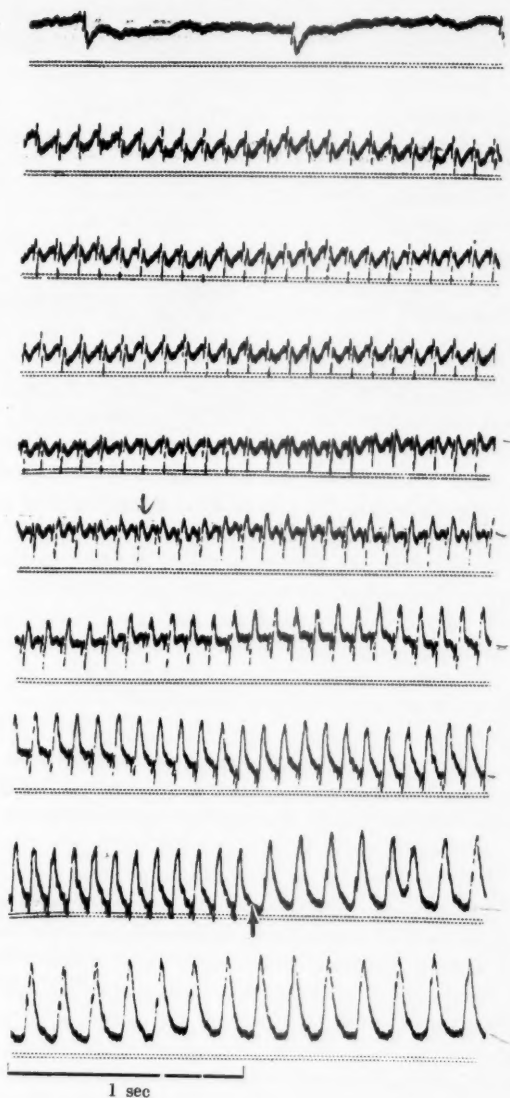Fig. 9. Repetitive stimulation of fornix. Active electrode saline filled pipette, depth 1.0 mm (dendritic field). Top line, 1/sec stimulation. Second and successive lines up to 9th, 10/sec stimulation. Stimulation ceases half way through the 9th line at arrow and after discharge continues as a series of negative potentials, resembling latter evoked potentials. Note gradual inversion of evoked potential. Time line 60 c. p. s.

how the effects indicated could be produced. It is not possible to say when an evoked potential becomes a driven spike though frequently, just before a seizure discharge 'break through', there are curious changes in latency. Sometimes these occur very abruptly, at other times they are progressive. One curious point noted was that if the after-discharge succeeded a slow tetanus (but was evident during stimulation) the frequency of the after-discharge was always slightly slower than the frequency of the tetanus (see Fig. 9). On the other hand, when then seizure discharge 'broke through' during slow stimulation it was often faster than the tetanus. Discharges following rapid tetanus (over 15 or 20/sec) bore no such relationship to the stimulus frequency. The inversion of the evoked potential is most marked in the dendritic layer. Near the surface it may also be seen, though it is less obvious. Nevertheless, inspection of the evoked potential during a slow repetitive stimulation allows one to predict when a seizure will occur or when it will follow the termination of the tetanus. The process of inversion thus appears to bear a fundamental relationship to the onset of seizure discharges.

Fig. 10 illustrates the inversion effect at three levels in the hippocampus. The record has been taken on a slowly moving film so that not only the inversion effects but also the slow potential shift which accompanies them have been recorded. The focal electrode in this case is a glass pipette filled with Ringer's solution and both the focal and reference electrodes are coupled through silver-silver chloride junctions to the D. C. amplifier. The reference is a cotton wick on the hippocampal surface. Stimulations at 1 per second is followed by 10/sec stimulation for the times indicated by the black bars. The baseline shift during stimulation is seen to be somewhat irregular, but generally in a positive direction. However, during an after-discharge it is clear that the surface layers become positive and the dendritic layer negative. On occasion these slow potential shifts continued for some time after the seizure discharge had ended. Their amplitudes varied up to a maximum of about 3 mV. It appears that a voltage is generated across the cell layer during a seizure discharge.

Vector analysis and evidence for ephapsis. DONALDSON and MATTHEWS (1955) proposed a vector technique for detecting the direction of nerve impulses. Since the uniform orientation of hippocampal cells suggested that the method might be applicable, a similar technique was tried in order to evaluate the direction of conduction within the pyramids.

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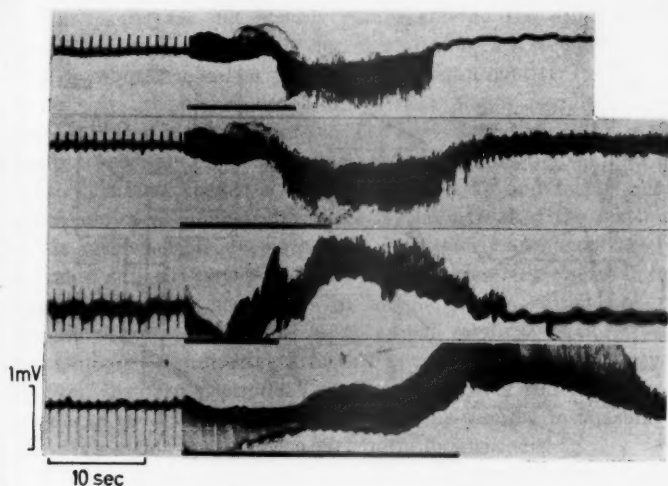oltage is

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Fig. 10. D. C. recordings before, during and after seizure discharges. First two records 1/sec stimulation, followed by 10/sec until after discharge commences. Recording electrode 0.2 mm below surface. Reference on surface. Third record: at 0.7 mm depth. Fourth record: at 1.0 mm depth. Note, that slow potential shift is in opposite direction below cell layer and begins at the time of inversion of the evoked potential. Calibrations 10 sec and 1 mV. Duration of stimulation indicated by solid line.

Principle. An oscillographic record of potential changes usually displays voltage along one axis against time represented along the other axis. When two simultaneously occurring signals have to be compared they may both be recorded against time with two separate oscilloscope beams as shown in Fig. 11 A. A more sensitive way to display temporal discrepancy is to record one of the signals against the other. One of the signals is recorded along the horizontal axis and the other signal along the vertical axis (Fig. 11 B). Thus at any instant the position of the oscilloscope beam gives the amplitude and sign of each of the signals as the x-coordinate and y-coordinate respectively. The time course may be registered if the beam brightness is modulated at a known frequency. Such a vector record can always be dissociated into two conventional voltage-time curves.

Methods. The electrodes used for vector recording were made of enamelled nichrome wires (no. 36) sheared at the tip and embedded in acrylic resin to the desired separation. The electrode system consisted of three electrodes. The tips described a right angled triangle either in a horizontal plane or in a vertical plane as shown in Figs. 11 and 12. The electrode that was the apex of the vertical triangles was thrust into the hippocampus so that its tip was in the dendritic field, the other two electrodes rested on the surface. With the electrodes in place 60
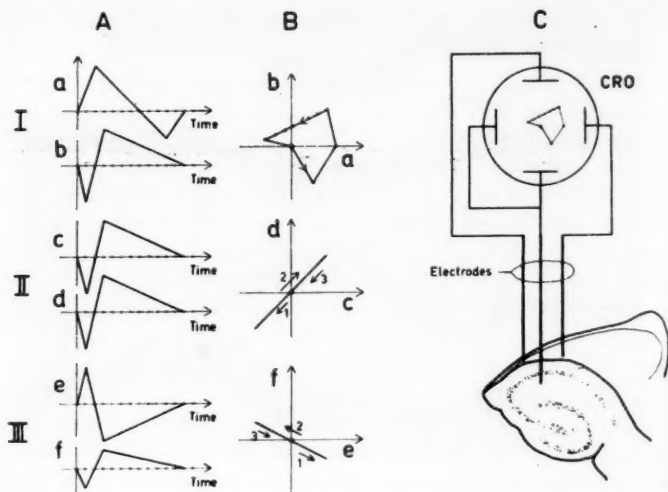
Fig. 11. Schematic diagrams of vector recording. I, shows two dissimilar signals *a* and *b* simultaneously recorded, A in the conventional way with amplitude against time, B against each other: *a* along the ordinate and *b* along the abscissa. If the two signals are exactly in phase and of same amplitude *i. e.* they are identical as in II, plotting them against each other results in a straight line in the first and third quadrants 45° to the abscissa (II B). Differences in amplitude will give another angle. Temporal differences will give loops. III shows an example where the signals *e* and *f* are of opposite polarity, exactly 180° out of phase which gives a straight line in the second and fourth quadrants. Since the amplitudes of the signals are unequal the angle is not 45°. C shows diagrammatically the electrode arrangement on the hippocampus as in Fig. 12 B. The electrode deep in the dendritic layer is in this case the common electrode. The signals displayed along the y-axis of the cathode ray oscilloscope, CRO, is thus recorded between the deep electrode and the left surface electrode while the signals displayed along the x-axis are recorded between the deep electrode and the right surface electrode.

c. p. s. AC was lead into the preparation and its vector adjusted to be a 45° straight line. Since this could easily be achieved there seems good reason to believe that whatever polarization occurred in the recording conditions was fairly symmetrical and did not significantly affect the shape of the vector record.

In the hippocampus, the resulting potential displayed proved a highly sensitive method for detecting temporal differences between electrical events occurring at the independent electrodes but rather impracticable for the analysis of direction of propagation since two vectors in the three planes of space and their subsequent analysis is about as cumbrous as the comparison of recordings between three pairs of electrodes in three planes.

The results are not strictly comparable to the other records since they were obtained under nembutal before it was realized that nembutal modifies the responses significantly. In Fig. 12 vectors are shown, recorded in vertical planes parallel to the fimbria and at right angles to it. The first deflection (1st 4 msec) seems to correspond to the fibre potential of the fimbria and alveus. This is succeeded by a loop, directed up and to the right, which indicates a phase difference in the arrival of the response at the surface electrodes both antero-posteriorly and transversely. Its main axis shows that it corresponds to the deep electrode becoming somewhat negative with respect to the surface. When stimulated repetitively the hippocampal response changes and this loop grows, indicating a further increase in the relative negativity of the deep electrode. The change following this loop corresponds to the later components of the hippocampal response and shows increasing relative positivity in the dendritic field.

When a seizure discharge is induced the vector shows many irregular loops (Figs. 12 and 13) which, however, may become rather regular as the discharge reaches the characteristic sinusoidal pattern of the electroencephalogram; then becoming desynchronized once more during the phase of seizure bursts at the termination of the discharge. Following the main after discharge spontaneous and driven spikes may be recorded. Examples are seen in Fig. 12. The spontaneous spikes are evidently exactly in phase at the recording electrodes. The driven spikes on the other hand are almost 180° out of phase recorded in a plane at 90° from the plane of the spontaneous spike. This was not as regular as the illustration suggests but they were sufficiently frequent to make it plausible that the electrode separation (2 to 3 mm) was about equal to half the wavelength of a wave travelling from front to back across the surface of the hippocampus at about 10 cm/sec.

The characteristics of the seizure records strongly suggest that abnormal neuronal pathways or random neuronal excitation is occurring during certain phases. Attempts to see whether specific pathways were involved in the spread of discharges indicated that seizure discharges in the hippocampus can be propagated across cuts. For example, when the cuts shown in Fig. 7 were extended deeply into the hippocampus (2 mm) so that all possibility of normal afferent connections to the 'island' were excluded seizure discharges induced in the island spread from it to adjacent hippocampus and *vice versa* presumably by a kind of

Fig. 12. Vector records of evoked potentials and seizure discharges. Diagrams of dorsal view of hippocampus at left indicate recording positions, with anterior represented toward the top of the page and lateral to the left. Arrow indicates point of stimulation. Dots represent surface electrodes, crosses electrodes deep in the dendritic layer. A, vertical deflexions recorded between posterior dot and cross and the horizontal deflexions recorded between anterior dot and cross. B, vertical axis medial dot to cross. Horizontal lateral dot to cross. C, vertical axis antero-medial dot to lateral dot, horizontal posterior dot to lateral dot. Beam brightness modulated at 500 c. p. s. Diagrams at right show direction of deflexions in evoked potentials. Negative on common electrode indicated up and to right. A: 1, evoked potential; 2, tetanic potentiation; 3, post-tetanic potentiation. B: 1, evoked potential; 2, tetanic potentiation; 3, initiation of seizure; 4, 5, spontaneous spikes following seizure. C: 1, 2, 3, driven spikes following seizure.

Fig. 13. Seizure discharge recorded from the hippocampus. Amplifiers and cathode ray tube connected to record vector (see text). Moving film. The common electrode is in the layer of pyramidal dendrites, the other two on the alveus. The upper record shows increasing synchronisation and the lower subsequent asynchronous discharges just before termination of the activity.

ephaptic mechanism. They were attenuated or abolished by inserting slips of glass in the cuts. More surprisingly, single shocks inside the island excited responses outside and *vice versa*. The latency of these was long and variable. Thus we obtained some evidence that the responses could traverse cuts under pathological conditions.

Discussion.

The earliest response to single shock stimulation of the fornix, occurring near the surface of the hippocampus, is not immediately followed by a response in the layer of cell bodies. Consequently it does not appear to invade the somata and, if it is an antidromic volley, it must be blocked near the axo-somatic junction. Since section of the alveus does not prevent a large post-synaptic response from the hippocampus, this small potential does not concern us here.

The sequence, position and duration of the recorded potentials accords well with the path proposed by GREEN and ADEY (1956). Thus a sharp response of 1.5 msec latency in the gyrus dentatus agrees with a first synaptic relay. A large response with latency of 4 to 5 msec and duration of well over 20 msec, accords with temporally dispersed activity or repetitive firing in second order neurones among the hippocampal pyramids. The axons of the granule cells form synapses with the pyramidal cells, near the junction of the somata and apical dendrites (CAJAL 1911; LORENTE DE NÓ 1934) and provide a straight-forward anatomical connection between pyramids and granule cells.

The dendritic layer of the hippocampus is remarkably homogeneous. It contains almost no neurone cell bodies. The most numerous axons therein are derived from the temporo-ammonic tract. These axons must have been separated from their cell bodies in the majority of our experiments. Consequently these axo-dendritic connections are not relevant to the present paper.

Possibly fibres reach the dendrites from the spheno-cornual bundle ('colonne horizontale') by the path described by GEREBTZOFF (1939) or, possibly, through the psalterium. These paths were cut surgically without any obvious effects and we have therefore disregarded them also.

Thus the only remaining afferent nervous structures which concern us here are the delicate axons of the granule cells and axon collaterals from the pyramidal cells. Since the latency of the main deflexion indicates at least a second order synapse and this deflexion is not abolished by cutting the fibres of the alveus we cannot have been dealing with a first order synapse in which the afferent fibres were derived from axon collaterals. It is concluded that the synapse is between the axons of the granule cells and the hippocampal pyramids at the junction of the cell bodies and the apical dendrites. We also concluded that, below this synaptic level, we are dealing only with the apical dendrites and we infer that electrical activity which may be localized to this layer is attributable to the effects of electrical changes in the dendrites themselves.

Since movements of the reference electrode did not change the shape of the evoked potentials, negativity at the focal electrode shows that the net current flow is inward through cell membranes and positivity net outward current. For reasons given in detail by TASAKI, POLLEY and ORREGO (1954), it is assumed that these changes occur close to the focal electrode. This conclusion is greatly strengthened by the large gradients which could be recorded over distances as small as 0.1 mm (Fig. 4). Local current flow is initiated in the pyramidal cells at the synaptic level discussed above. The inward current detected here (Fig. 2) is accompanied by corresponding outward current from the distal dendritic regions. There is no sign of a subsequent gradual shift of inward current to the dendrites, from which a rather pure positive response is recorded.

Consequently, if the evoked potential is chiefly due to summated pyramidal cell action potentials, somatafugal propagation in

most dendrites must be blocked. On another basis EULER and
RICCI (1958) reached a similar conclusion with respect to the neo-
cortical dendrites of the cat auditory area. The concept of a con-
duction block is obviously not necessary if the evoked potential
is merely the envelope of events which are only electrotonically
conducted: pre-potentials, for example. Since, however, large post
synaptic responses have been recorded in the fornix following
hippocampal stimulation (GREEN and ADEY 1956) it is likely that
propagated action potentials contribute largely to the evoked
potential.

The rather small signs of inward current in the cell body layer
detected in focal records is explained readily, since this layer is
actually a mixture of tightly packed cell bodies and dendritic
shafts so that outward and inward currents at this level tend to
cancel each other. The dendritic layer, in contrast, is free of neu-
rone cell bodies and summated outward current is recorded here.
However, the curves of Fig. 4 clearly show that the region framed
by the largest gradients and therefore of largest current flow is
the layer of pyramidal cells, as discussed in the results.

The families of curves in which amplitude is plotted against
depth show remarkably persistent electrical events at all levels
within the pyramidal layer. The absence of signs of propagation
from soma to axon is explicable in several ways, however. The
axons are very thin and surrounded by far more numerous basal
dendrites. It is also possible that the somata and proximal axonal
segments depolarize virtually simultaneously so that propaga-
tion could only be detected at some distance from the cell. The
somewhat unlikely alternative of a second block between the soma
and axon as well as between the synaptic level and dendrites is
contradicted by the observation of postsynaptic responses in the
fornix (GREEN and ADEY 1956).

There are some discrepancies between our findings and those of
CRAGG and HAMLYN (1955). These can best be explained by in-
dicating that they stimulated within the substance of the hippo-
campus attempting to excite the granule cell axons specifically
and that their experiments were performed under nembutal an-
aesthesia which we found to alter the response at the level of the
pyramidal cells.

Some explanation is needed for the small notch on the positive
wave recorded from the dendritic layer. It is hard to determine
its exact latency from our records but this is probably not much

greater than that of the main deflexion. There is no evidence that the notch is propagated. If it represented an interval between preferred firing times of the pyramidal cells it should be noted with opposite sign in the direct records obtained at the synaptic level. Since it is not clearly seen there, the inference is either that it is masked or that it has no corresponding phase at the soma layer. Since it occurs somewhat after the main deflexion and seems to be most evident at about 0.8 to 1.1 mm depth it is possible that axon collaterals which terminate at about this level, cause a small inward current here, the delay being due to conduction time in the collaterals.

The appearance of inward current in the dendritic layer which follows repetitive stimulation is often, though perhaps not always, initiated by the deepening of this small notch, which then becomes a clear negative potential (Fig. 9). This suggests that the notch is an active phenomenon and is not due to a passive change in rate of firing.

We do not consider that the growth of this notch is the only explanation of the inward current which appears in the dendritic layer after repetitive stimulation. If the soma is fired at an increased rate the excitability of the dendrites may change so that the position of a block may shift. Either or both of these explanations would account for the dramatic changes seen in Fig. 9.

There seems to be an association between the inversion of the dendritic layer response and the initiation of a seizure discharge. When the dendritic layer response to repetitive stimulation becomes monophasically negative, indicating inward current through the dendritic membranes, an after-discharge may be predicted, as described in results. This suggests that inward current in the dendritic layer is fundamentally related to the seizure discharge mechanism. The potentials of the after-discharge, like the evoked potentials which precede it, are also monophasic negative. Thus a state of hyperexcitability now exists in the dendritic layer. Where stimulation is more prolonged this excitability reaches a state in which it is no longer restrained by the refractory phase following each response, so that seizure spikes appear between the responses and interact with them. Similar results were described by GREEN and ADEY (1956).

The vector record at the onset of a seizure shows that electrical discharges follow no orderly time sequence but appear to be quite random. As the discharge progresses the vector record may be-

come quite regular (Fig. 13) showing that a high degree of syn-chronisation has occurred between adjacent regions a few milli-meters apart.

Slow potential shifts have been observed during seizure dis-charge in the neocortex by VAN HARREVELD and STAMM (1954) and by DONDEY and SNIDER (1955). LIBERSON and CADILHAC (1953) saw them in the hippocampus during after-discharges but noted that the sign of the potential shift varied from one experi-ment to another although its magnitude seemed to be correlated with the after-discharge. Our own findings offer an explanation for the latter curious observation for the sign of the shift depends on the level of recording with respect to the somata. Since the slow potential shift has a magnitude of several millivolts and may last a minute or more, it is likely that it can modify the excitabil-ity of the neurones.

Vector records obtained in two perpendicular planes (Fig. 12) indicate that a seizure spike may often sweep slowly as a wave of activity over the hippocampus in an anterior-posterior direction with a velocity of about 10 cm p. s.

These peculiar horizontal movements do not accord with any known anatomical paths and are not related to any temporal features of the ordinary evoked potential. It is concluded that when pathological electric activity occurs a new kind of trans-mission is initiated in the dendritic layer, which at this time is the region of inward current as shown in the D. C. records (Figs. 9 and 10). That transmission of seizure potentials occurs through abnormal pathways accords with the findings of ROSENBLUETH, BOND and CANNON (1942) on the isolated occipital cortex, the findings of GERARD and LIBET (1940) in the frog and may be com-pared to BREMER's experiments (1941) on the strychninized cord in which it was found that two segments severed from each other still beat in unison if contact was permitted. It is further supported by the evidence presented here that the discharge can travers cuts and may explain transmission of discharges from hippocampus to the temporal lobe after severing all known pathways as observed by GREEN and SHIMAMOTO (1953). Cuts may create artificial synapses in a similar sense to those described by GRANIT, LEK-SELL and SKOGLUND (1944). The establishment of an artificial synapse appears to be related to a partial depolarization following injury with a consequent increase in excitability. This is very similar to the state created in the dendritic layer by repetitive stimulation.

It is believed that the evidence presented here suggests extreme caution in the interpretation of records from closely spaced electrodes within the nervous system, for a condition of block which is to some extent reversible may lead to large changes in evoked potentials recorded in this way. These changes may have little or nothing to do with the number of neurones excited.

Summary.

1. Responses were evoked in the hippocampal pyramidal and granule cells by stimulation of the dorsal fornix.

2. Current flow at different instants after the shock and from different positions within the hippocampal laminae is described.

3. Evidence is presented to show that the evoked potential is not propagated in the apical dendrites of the pyramidal cells.

4. After repetitive stimulation, or after injury, signs of inward current may be detected in the layer of apical dendrites.

5. This inward current is associated in some way with seizure discharges.

6. Seizure discharges may propagate by routes which do not correspond to normally conducting pathways.

References.

ADRIAN, E. D., J. Physiol. 1936. *88*. 127.

BREMER, F., Arch. int. Physiol. 1941. *51*. 211.

CAJAL, S. RAMON Y., Histologie du système nerveux de l'homme et des vertébrés. Paris, A. Maloine, 2 vols. Trans. by L. Azoulay. 1911.

CAJAL, S. RAMON Y., Studies on the cerebral cortex (limbic structures). London, Lloyd-Luke. Trans. by L. M. Kraft. 179 pp. 1955.

CRAGG, B. G. and L. H. HAMLYN, J. Physiol. 1955. *129*. 608.

CRAGG, B. G. and L. H. HAMLYN, J. Physiol. 1957. *135*. 460.

DONALDSON, P. E. and B. H. MATTHEWS, J. Physiol. 1955. *129*. 35 P.

DONDEY, M. and R. S. SNIDER, EEG Clin. Neurophysiol. 1955. *7*. 265.

EULER, C. VON and G. RICCI, Neurophysiol. 1958. In press.

GERARD, R. W. and B. LIBET, Amer. J. Psychiat. 1940. *96*. 1125.

GEREBTZOFF, M. A., J. belge Neurol. Psychiat. 1939. *39*. 205.

GIBBS, F. A. and E. L. GIBBS, Arch. Neurol. Psychiat., Chicago, 1936. *35*. 109.

GRANIT, R., L. LEKSELL and C. R. SKOGLUND, Brain 1944. *67*. 125.

GREEN, J. D. and W. R. ADEY, EEG Clin. Neurophysiol. 1956. 8. 245.

GREEN, J. D. and A. ARDUINI, J. Neurophysiol. 1954. 17. 533.

GREEN, J. D. and X. MACHNE, Amer. J. Physiol. 1955. 181. 219.

GREEN, J. D. and T. SHIMAMOTO, Arch. Neurol. Psychiat., Chicago, 1953. 70. 687.

HARREVELD, A. VAN and J. S. STAMM, J. Neurophysiol. 1954. 17. 506.

JUNG, R., Arch. Psychiat. Zeitschr. Neurol. 1949. 183. 206.

KAADA, B. R., Acta physiol. scand. 1951. 24. Suppl. 83. 285 pp.

LIBERSON, W. T. and G. CADILHAC, EEG Clin. Neurophysiol. 1953. 5. 320.

LORENTE DE NÓ, R. J., J. Psychol. Neurol., Lpz. 1934. 46. 113.

RENSHAW, B., A. FORBES, and B. R. MORISON, J. Neurophysiol. 1940. 3. 74.

ROSENBLUETH, A., D. D. BOND and W. B. CANNON, Amer. J. Physiol. 1942. 137. 681.

TASAKI, I., E. H. POLLEY and F. ORREGO, J. Neurophysiol. 1954. 17. 454.

Co-ordinated Changes in Temperature
Thresholds for Thermoregulatory Reflexes.

By

CURT von EULER and ULF SÖDERBERG.

Introduction.

The hypothalamic thermodetectors are believed to exert a
tonic influence on the relay systems of the brain stem from which
much of the activity of the organism is governed. Raising the
temperature of the anterior hypothalamus may depress both
motor and cortical activity and a fall in temperature of this
region enhances the activity of the cortex and the motor system.
Since increased activity means increased heat production this
mechanism tends to serve the maintenance of temperature
homeostasis. The general level of activity, thus, seems to be one
of the factors regulating body temperature. Nevertheless the
body temperature does change with different levels of activity.
Sleep and wakefulness, emotions and muscular activity are well
known to be accompanied by changes in body temperature. It is
relevant to ask whether these drifts in body temperature are signs
of a failing temperature regulation or, contrarily, whether they are
the result of some regulatory mechanism. The latter alternative
contains the hypothesis that the body temperature may not be
regulated to a constant level but to an optimal level which may
vary with the situation. In fact, NIELSEN (1938) has shown that
the rise in body temperature during muscular exercise is the result

of a precise regulatory process and not due to failure of the temperature regulation. He found the rise in temperature to be directly proportional to the intensity of the work but uninfluenced by wide variations in the environmental temperature and concluded that the 'body thermostat' could be 'set' to different levels under different circumstances. His suggestion that 'setting the body thermostat' to a higher temperature level during work would improve performance has been amply verified by ASMUSSEN and BÖJE (1945).

No analysis of the mechanisms involved in this 'setting' has been presented. We have approached the question by studying the hypothalamic temperature levels at which several heat-dissipating, heat-preserving and heat-producing reflexes appeared and by which means these hypothalamic threshold temperatures could be shifted to new temperature levels. Co-ordinated changes in threshold temperatures for these thermoregulatory reflexes have been elicited in response to several different stimuli. The body temperature changed correspondingly. This is believed to represent a 'resetting' of the body thermostat to the needs at any particular time.

Methods.

Detailed description of methods and technical procedures is given in a previous communication (EULER and SÖDERBERG 1957). Adult male and female cats and rabbits were used. The rabbits were anaesthetized with urethane (1.5 g per kg body weight) or chloralose-urethane (10 per cent urethane in 1 per cent chloralose, 5 ml per kg body weight). The cats were anaesthetized with sodium pentobarbital ('Nembutal', Abbott, 40 mg per kg body weight). The anaesthesia was in some cases complemented with small doses of 'Pentothal', Abbott, or urethane. *Encéphale isolé* preparations of cats were also used.

The impulse pattern generated by single muscle spindles was recorded from fine filaments dissected from the dorsal roots of L7 or S1 according to the description of GRANIT and KAADA (1952), (*cf.* also GRANIT 1955). When the simultaneously recorded mechano-myogram at its highest sensitivity showed no deflections, changes in muscle spindle activity were interpreted as changes in gamma motor activity.

Changes in cutaneous blood flow were estimated from thermo-electrical temperature measurements of skin surface or records of venous outflow according to FOLKOW, STRÖM and UVNÄS (1949). Blood pressure was recorded with a strain gauge manometer. Hypothalamic and rectal temperatures were also followed thermo-electrically.

Shivering was recorded with gramophone pick-ups resting on the body surface and coupled to one of the channels of the electroencephalo-

graph. The electrocorticogram was registered from epidurally implanted electrodes with a six channel electroencephalograph (Offner, type A). Respiration, oxygen consumption and infusion rates were also recorded.

Results.

Heating the anterior hypothalamus generally provoked vasodilatation of the cutaneous vessels of ears and pads both in cats and rabbits, whereas constriction of these vessels was provoked by such stimuli which induced an 'arousal reaction' seen behaviourally and judged from the electrocorticogram. Fig. 1 is obtained from an experiment on a rabbit under light urethane anaesthesia. Hypothalamic heating was introduced during a period of slowly increasing cutaneous vasoconstrictor tone of the ear. The vasoconstrictor tone was not inhibited until the temperature of the heating electrodes had reached 39—40° C when a progressive vasodilatation started. While the hypothalamic temperature was maintained at this level a prolonged repetitive stimulation within the midbrain tegmentum in the vicinity of the red nucleus caused the ear skin temperature to fall again. In order to overcome this vasoconstriction the recorded hypothalamic temperature had to be raised to 41—42° C. Thus the threshold temperature for inhibiting cutaneous vasoconstrictor tone was raised by at least 1° C by the stimulation of the midbrain tegmentum. Immediately after cessation of the hypothalamic heating the ear skin temperature dropped again, indicating vasoconstriction. These pronounced changes in the cutaneous vasomotor activity of the ears were accompanied by very little changes in the skin temperature of the thigh or of the back. The ears of the rabbit are, of course, powerful effector organs in the temperature regulation (KÖNIG, 1943; HENSEL, 1952) while the constancy of skin temperature of the trunk and the thigh shows that the vessels of these major parts of the skin participate to a much less extent as regulatory of heat dissipation. Close inspection of the rectal temperature record reveals a slight fall during the period of vasodilatation in the ear. At the end of the hypothalamic heating there is a slight rise in body temperature which stops after the cessation of the electrical stimulation of the midbrain tegmentum. The uppermost curve gives the respiratory frequency which was increased during the period of stimulation. It is well known that the 'arousal reaction' in the rabbit is accompanied by an increased respira-

tory frequency, here referred to as 'emotional polypnea' to dis-
tinguish it from 'heat polypnea'. The emotional polypnea seen
in the figure was of the shallow type which does not tend to cause
hypocapnea. ALBERS, BRENDEL and USINGER (1956) have shown
a decrease in oxygen tension and hypercapnea during periods of
emotional polypnea in dogs. In most cases heating the anterior
hypothalamus caused vasodilatation of the ears and the pads.
Excessive heating, however, caused periods of vasoconstriction
or decreased rate of vasodilatation corresponding to the periods
of high muscle spindle activity and emotional polypnea. Fig. 2
demonstrates the rivalry between inhibition and excitation on
the vasomotor neurons of the ear skin vessels. The 'interval
pattern' of muscle spindle activity (EULER and SÖDERBERG 1957)
from a single muscle spindle of the gastrocnemius muscle is re-
corded concomitantly. The muscle spindle discharged in bursts
with a duration of somewhat less than one minute. At the end
of each burst the discharge frequency dropped as indicated by
longer intervals, *i. e.* longer deflections. In this case the local
hypothalamic heating did not influence the muscle spindle pat-
tern. However, it did have an inhibitory action on vasoconstrictor
tone. The hypothalamic heating started during a period of high
muscle spindle activity. No effect on the ear skin temperature
was recorded until the muscle spindles came to a silence. Then
vasodilatation appeared, but was interrupted at each burst of
high muscle spindle activity. The ear temperature remained un-
changed as long as the muscle spindle activity was high. When
the discharge frequency decreased, vasodilatation started again.
During the next burst of muscle spindle activity the ear tem-
perature curve turned, indicating vasoconstriction. At the cessa-
tion of the hypothalamic heating the ear temperature dropped
stepwise towards the initial level. Thus, the threshold temperature
for the inhibition of cutaneous vasoconstrictor tone seems to be
increased during periods of high muscle spindle activity.

Fig. 1 is a good illustration of how little the temperature of the
main skin areas is influenced by our experimental procedures.
It is, thus, unlikely that the described effects are mediated by
the peripheral thermoreceptors of these skin areas. The surface
temperature of the ears, however, varies greatly with different
thermoregulatory needs. The question then arises whether the
thermoreceptors of the ear skin exerts an influence on the ther-
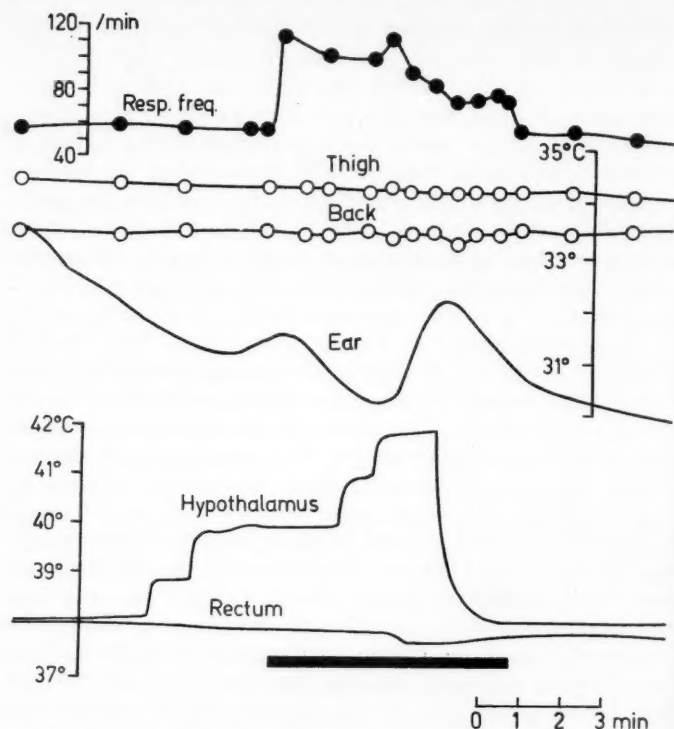moregulatory events.

Fig. 1. Rabbit, 1.7 kg. Urethane. Effects of hypothalamic heating and brain stem stimulation. Top tracing, with calibration to the left, shows respiratory frequency. Tracings labelled 'Thigh' and 'Back' and the continuous record marked 'Ear' give the surface temperature of these skin areas; calibration to the right. The continuous temperature records from 'Hypothalamus' and 'Rectum' have their calibration to the left. Heavy black line indicates period of stimulation 300 per sec of midbrain tegmentum in the vicinity of *nucleus ruber*.

Heating or cooling the ears of the rabbit never caused any detectable difference in the threshold temperature for eliciting and inhibiting shivering. Further, when testing out the threshold temperature for cutaneous vasomotor reactions this was found to be the same regardless of whether the threshold for vasoconstriction was studied on a warm ear or whether the threshold for vasodilatation was studied on a cold vasoconstricted ear. We may therefore conclude that the effects studied here are not moderated by peripheral thermoreceptors.

Fig. 2. Rabbit, 2.2 kg. Urethane-chloralose. Intervals between action potentials from a dorsal root filament of a single gastrocnemius muscle spindle recorded with an electronic ordinate recorder (short deflections — short intervals, long deflections — long intervals). Hypothalamic temperature (H. T.) and surface temperature of the ear (E. T.) simultaneously recorded. For further explanation, see text.

In order to obtain reliable threshold temperatures for changes in total heat production, oxygen consumption was measured continuously with a Krogh spirometer of a size suitable for cats and rabbits. The effects of changes in body and hypothalamic temperatures were studied during various changes in wakefulness. Such an experiment is illustrated in Fig. 3 where the whole experiment is graphically reproduced in *A* and some of the original spirometer tracings are reproduced in *B*. In *A* the upper curve represents the oxygen consumption and the lower curve the rectal temperature. The periods of shivering, hypothalamic heating and stimulation by twisting the pinna are indicated below the curves. At the beginning of the records of Fig. 3 the cat had not yet reached thermo-balance and was still shivering although the rectal temperature was as high as 40.0° C. A cat under light 'Nembutal' anaesthesia often exhibits varying degrees of hyperthermia (EKSTRÖM 1951). Hypothalamic heating immediately stopped shivering and decreased the oxygen consumption. (There is a time lag between the oxygen consumption and the spirometer tracing largely due to the volume of the tubings and the valve.) On cessation of hypothalamic heating oxygen consumption returned to its previous level and shivering reappeared. This short decrease in heat production was not reflected in the record of rectal temperature, which continued to rise to a level of 40.2° C at which oxygen consumption decreased spontaneously and shivering stopped with a concomitant fall in rectal temperature. Twisting the pinna accelerated oxygen consumption and provoked shivering both of which were inhibited by hypothalamic heating. During the period of elevated hypothalamic temperature twisting the pinna did not provoke a marked shivering but a significant
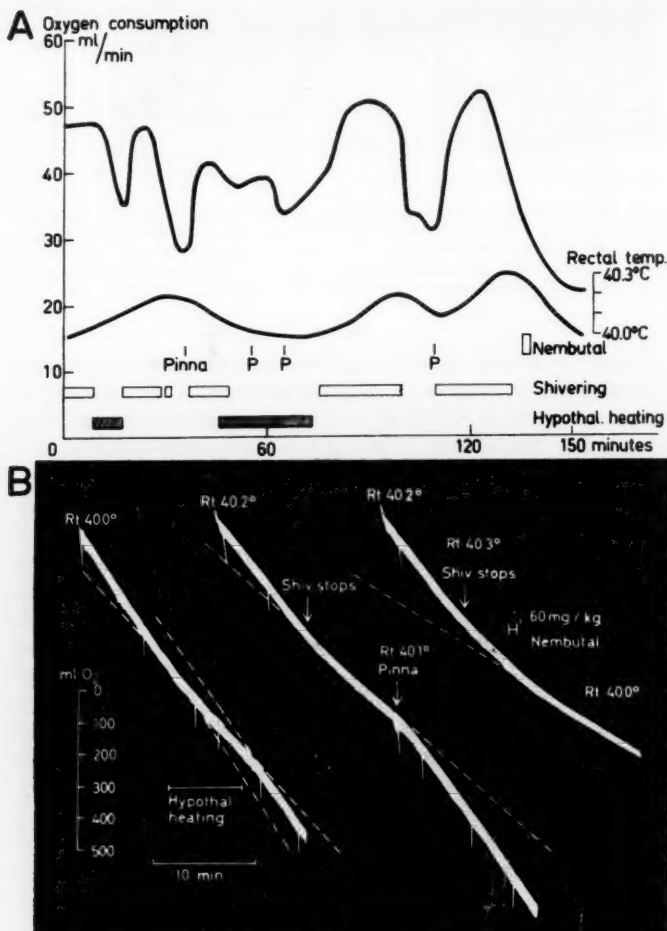
Fig. 3. Cat, 3.2 kg. 'Nembutal'. *A*. Upper curve: oxygen consumption constructed from the original spirometer records; calibration to the left. Lower curve: rectal temperature; calibration to the right. Below the curves marks indicate twisting the pinna, injection of 'Nembutal' i. v. and periods when shivering was observed and heating of anterior hypothalamus. *B*. Samples of the original spirometer tracings. Between first and second tracings, records from a period of 75 min. are omitted. Second and third tracings are consecutive.

increase in oxygen consumption indicating that this stimulus opposed the effect of hypothalamic heating. At the cessation of the heating the shivering thus started again, drove oxygen consumption to high values until the previous temperature threshold for spontaneous inhibition was reached, and the rectal temperature turned. After a fall of the body temperature of $0.1°$ C, shivering was provoked by a pinna reflex whereby the body temperature was driven up to $40.3°$ C before shivering ceased. The experiment was ended by injecting a large dose of 'Nembutal' in order to obtain a value for basal metabolic rate according to BRENDEL, KOPPERMANN and THAUER (1954). This experiment clearly demonstrates the acceleration of heat production and provocation of shivering by the pinna reflex and the suppression of these effects by local hypothalamic heating. Gently stroking the back of the cat or lifting the skin of the neck and shoulder region were accompanied by synchronization of cortical activity, dilatation of skin vessels of the ear and inhibition of shivering. As a consequence a drop in body temperature was obtained. Similarly it was often observed that spontaneously appearing synchronous activity in the cortex was followed by inhibition of vasoconstriction and shivering.

As the 'arousal reaction' is characterized by increased activity in adrenergic systems (DELL, BONVALLET and HUGELIN 1954) a natural step was to study the effect of adrenaline and noradrenaline on threshold temperatures for thermoregulatory reactions. The effect of single injections did not permit estimations of changes in thresholds. For this reason and to imitate physiological conditions, the catechols were administered by slow rate infusion. Such an experiment is shown in Fig. 4 which compares a record of shivering with electrocorticograms from four cortical regions. The cat under light 'Nembutal' anaesthesia was apparently in thermo-balance with no shivering but with some vasoconstrictor tonus in the vessels of the ears (surface temperature $26.2°$ C) and pads (surface temperature $31.5°$ C). The EEG showed frequent spindle bursts characteristic for a drowsy cat under light 'Nembutal' anaesthesia (A). At the arrow intravenous infusion of noradrenaline at a rate of 1 μg per min. was introduced. After two minutes, $i.\,e.$ 2 μg, shivering appeared (uppermost tracing) and EEG showed less frequent spindle bursts and some increase in low voltage high frequency activity. Slight local hypothalamic heating was now introduced in order to inhibit the shivering thus
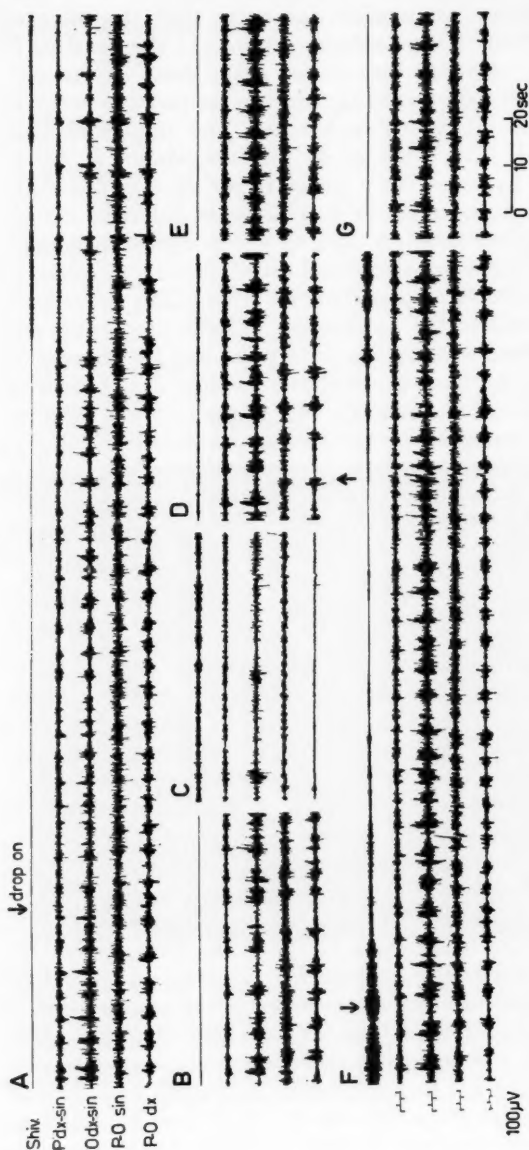
Fig. 4. Cat, 3.1 kg. The influence of noradrenaline infusion on EEG and threshold temperatures for inhibition of shivering by hypothalamic heating. *A.* Onset of noradrenaline infusion (1 µg/min.). *B.* Noradrenaline infusion as in *A* counteracted by hypothalamic heating (0.4 mA). *C.* No hypothalamic heating, increased rate of noradrenaline infusion (1.6 µg/min.). Note, shivering more intense than in right part of *A. D.* Noradrenaline as in *C.* Hypothalamic heating as in *B.* Note, shivering. *E.* Same as *D* but increased hypothalamic heating (0.45 mA). *F.* Increased rate of noradrenaline infusion (3.6 µg/min.). At ↓ increased hypothalamic heating (from 0.45 to 0.50 mA). At ↑ cessation of noradrenaline infusion. *G.* After cessation of hypothalamic heating. No hypothalamic heating. *P.* Parietal. *O.* Occipital.

provoked. In *B* this was accomplished by 0.4 mA. In *C* the hypothalamic heating was switched off and the rate of infusion of noradrenaline was increased to 1.6 μg per min. The shivering thus elicited was more intense than that seen in right part of *A* and the effect on the EEG was also more obvious. Hypothalamic heating necessary to abolish the effect of 1 μg per min. of noradrenaline did not suffice to counteract the effect of the higher rate of infusion (*D*) but 0.45 mA of heating was successful (*E*). Stepwise increase of rate of noradrenaline infusion had correspondingly to be followed by stepwise increase of intensity of hypothalamic heating. Thus 0.50 mA of hypothalamic heating was just able to check the effect of 3.6 μg of noradrenaline per min. (between arrows in *F*). Cessation of hypothalamic heating was immediately followed by the reappearance of vigorous shivering which again was abolished on cessation of the intravenous drop infusion of noradrenaline. Noradrenaline thus pushed threshold temperature for shivering to a higher level, *i. e.* in the same directions as that found for other 'arousing' stimuli.

It was surprising to find that adrenaline gave the same results as noradrenaline in the same doses and that there were no apparent differences between the effects of the two hormones on the threshold temperatures. In view of the results of DELL et al. (1954) and ROTHBALLER (1956) that adrenaline exerts a stimulating effect on structures in the higher brain stem, it was thought worth while to compare the effects of infusion of adrenaline and noradrenaline administered intravenously and intraarterially, close to the brain circulation. To obtain the same effects the same doses were, however, found necessary, regardless of the route of administration: into the internal carotid artery, the vertebral artery or the femoral vein.

Further attempt to localize the described effects of adrenaline was done in experiments with crossed circulation, where one of the forelimbs of the cat under experimentation was perfused by blood from another cat. This forelimb was thus disconnected from the circulation of its own body but the nervous connections were kept intact. Cutaneous circulation was measured both with thermocouples and by recording the venous outflow (FOLKOW, STRÖM and UVNÄS 1949). Adrenaline infusion into the general circulation of the recipient at low rate gave increased constrictor tone also in the cutaneous vessels of the perfused limb. This effect of adrenaline on the perfused vessels must have been mediated

(0.45 mA). *F*. Increased rate of noradrenaline infusion (3.6 μg/min.). At ↓ increased hypothalamic heating (from 0.45 to 0.50 mA). At ↑ cessation of hypothalamic heating. *G*. After cessation of noradrenaline infusion. No hypothalamic heating. *P*. Parietal. *O*. Occipital.
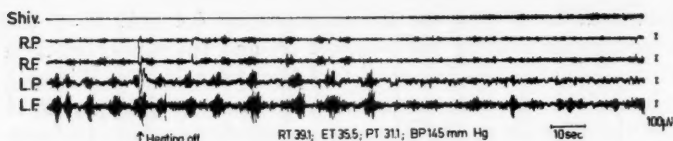
Fig. 5. Cat, 5.2 kg. 'Nembutal'. Hypothalamic heating synchronized the cortical activity, inhibited shivering and elicited cutaneous vasodilatation (surface temperature of ear, E. T. 35.5° C, of pad, P. T. 31.1° C). On cessation of the hypothalamic heating, at arrow, cortical activity was desynchronized and shivering started. Rectal temperature, R. T., at this moment 39.1° C and blood pressure 145 mm Hg.
R. Right; L. left; P. parietal and F. frontal.

by the vasomotor nerves. It is well known that cutaneous vasoconstrictor tone, which is of great importance for temperature regulation, is influenced by adrenaline. This experiment has revealed that this effect is partly central and caused not only by the action of adrenaline on the effector organs. A good correlation was often observed between the spontaneous variations in vasomotor tone of the perfused vascularly isolated limb and the cortical electrical activity. Attention may be drawn to the correlation between gamma motor activity and cutaneous vasomotor tone shown in Fig. 2, and between gamma motor activity and cortical activity demonstrated previously by EULER and SÖDERBERG (1957). An example of the relation between shivering and cortical electrical activity may be seen in Fig. 5.

It was frequently observed that late in the experiment shivering was less intense and not so readily obtained. This was commonly found to be true also for the muscle spindle activity. Administration of glucose restored the previous responsiveness. However, these effects of adrenaline and noradrenaline were probably not mediated by liberation of glucose, since adrenaline is more active than noradrenaline on gluconeogenesis (for references see U. S. VON EULER 1956). Furthermore, in our experiments, large doses of glucose did not change the character of responses to the catechols. These results have also been obtained in cats and rabbits after adrenalectomy.

Increased motor activity often puts a big load on the regulation of the blood gases. Carbon dioxide and oxygen are, of course, of critical importance for the excitability of central nervous structures. In the present experiments artificial changes in the composition of the inspired gases were induced and the effects on threshold temperatures were studied. The records in Fig. 6 are
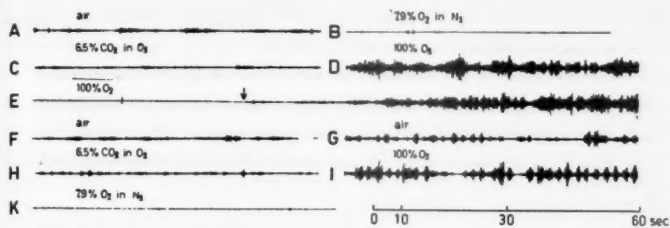
Fig. 6. Cat, 3.0 kg. 'Nembutal'. Records of shivering showing the effects of the composition of the inspired gas. $A—F$: Spontaneous breathing; $G—K$: forced breathing of 3.6 l per min. Record E, 100 per cent O_2 spontaneous breathing, starts during hypothalamic heating of 0.6 mA. Shivering is inhibited. At arrow the heating current is switched off and shivering is soon as violent as in record D.

obtained from such an experiment on a cat under light 'Nembutal' anaesthesia, which was shivering moderately when breathing air spontaneously (Fig. 6 A). Changing over to a gas mixture of 7.9 per cent oxygen in nitrogen, which caused a marked increase in tidal air, inhibited shivering completely (record B) concomitantly with a rise in blood pressure of 15 mm Hg. During breathing of 6.5 per cent carbon dioxide in oxygen, which gave even greater increase in ventilation, shivering was less intense than during air breathing (record C). When pure oxygen was inspired, shivering became much more violent as seen from record D. Local heating of the anterior hypothalamus could check this shivering. Higher temperatures had to be induced to inhibit shivering when the animal was breathing oxygen than when it was breathing air (record E). At the cessation of the hypothalamic heating, performed under oxygen, shivering often reappeared with somewhat increased intensity, probably due to the slight fall in body temperature during the period of hypothalamic heating. When shivering was inhibited by oxygen deficiency body temperature also dropped. The intense shivering during periods of oxygen breathing was regularly accompanied by a slight rise in body temperature.

The same gases were also tested under constant artificial respiration with the tidal air set at various levels. The same results as those under spontaneous breathing were seen under artificial ventilation (records $G—K$). It is difficult to find a common denominator for these responses. However, it seems likely that the peripheral chemoreceptors played a major part for the
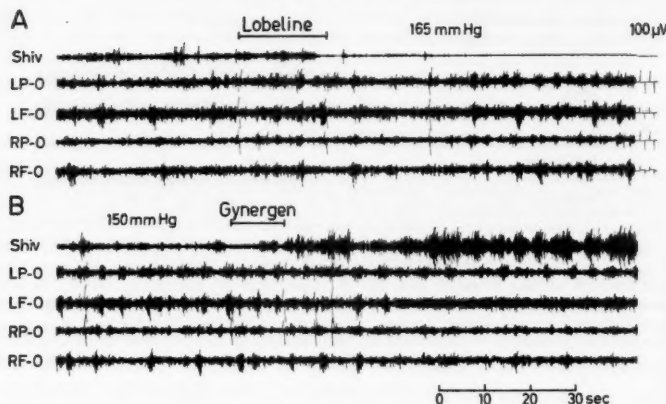
Fig. 7. Cat, 2.7 kg. 'Nembutal'. Effects of A 1 mg lobeline i. v. and B 0.1 mg ergotamine tartrate (Gynergen) i. v. on shivering (upper tracing in each set) and on EEG. Voltage calibration to the right in A 100 μV.

effects, since the thermoregulatory events tested were more sensitive to changes in oxygen tension than to changes in carbon dioxide. This view is supported by the finding that lobeline in small doses (0.3 mg per kg i. v.), which has a stimulating effect on the peripheral chemoreceptors (HEYMANS, BOUCKAERT and DAUTREBANDE 1931; U. S. VON EULER, LILJESTRAND and ZOTTERMAN 1939) readily inhibits shivering and thus has an effect similar to oxygen lack (Fig. 7 A).

Discussion.

All the effectors involved in thermoregulation are utilized also for other duties and show great variations in their activity to meet needs other than maintenance of temperature homeostasis. Their relative temperature thresholds and consequently the mutual order in which they are brought into action by the central and peripheral thermodetectors thus varies with the prevailing circumstances. Some of the changes in threshold observed might have served such a purpose. However, when shifts in threshold were followed by a shift in body temperature it has been regarded as a setting of the 'body thermostat' since it then indicates a co-ordinated change in thresholds of both heat dissipating and

heat producing reflexes. The temperature at which cutaneous vasoconstriction was inhibited, and the temperature at which shivering occurred, were raised by such procedures as electrical stimulation of the brain stem tegmentum, pinna reflexes, and nociceptive stimulation which all activated both motor systems (EULER and SÖDERBERG 1957) and the cortical electrical activity. These stimuli thus elevated the body temperature. Conversely, inhibitory reflexes lowered the threshold temperature for thermoregulatory events, resulting in a fall in body temperature. It was, however, difficult to change the body temperature more than a few tenths of a degree centigrade by these means and these experiments may be of greater relevance for the understanding of the influence of emotions on body temperature than for an interpretation of Nielsen's results. (Emotional hyperthermia, see e. g. VAN DER BOGERT and MORAVIC 1937, and KLEITMAN 1945.) The rate of adrenaline and noradrenaline secretion is closely related to the level of activity of man and animals (CANNON 1932; U. S. VON EULER, HELLNER-BJÖRKMAN and ORWÉN 1955; KÄRKI 1956) and is greatly increased during muscular work (HOLTZ, CREDNER and KRONEBERG 1947; U. S. VON EULER and HELLNER 1952; HOLMGREN 1956; KÄRKI 1956). Thus, it seems likely that adrenaline and noradrenaline are of great importance for the shift of the body temperature during muscular exercise.

In our experiments adrenaline infusion raised the body temperature. This was associated with changes in threshold temperatures of regulatory reflexes. These effects of adrenaline must have been at least partly mediated by central nervous structures since vasoconstriction was readily demonstrated in skin areas vascularly isolated by perfusion from another animal but with intact innervation.

An extensive literature deals with the calorigenic action of adrenaline and CANNON, QUERIDO, BRITTON and BRIGHT (1927) reinvestigated the effect of cold on adrenaline secretion. They concluded that "a disturbing heat loss evokes activity of adrenal medulla and that the extra output of adrenaline by hastening combustion, serves to protect the organism against cooling". In this study, however, we are only concerned with the effect of adrenaline on the nervous control of body temperature and will not enter into the discussion on chemical heat production. LUSK and RICHE (1914) found that subcutaneous adrenaline injections in dogs raised the metabolic rate due to an increase in muscular

activity. On p. 122 it was argued that this influence is not likely
to be mediated by the glucose level. From the finding of DELL
et al. (1954) and of ROTHBALLER (1956) that adrenaline has a
stimulating effect on the activating relays of the brain stem, one
would have expected to find greater sensitivity to adrenaline
when it was administered into the vertebral or carotid arteries
than when injected intravenously. The adrenaline 'arousal reac-
tion' of the cortex is difficult to obtain in animals under light
barbiturate anaesthesia. Temperature regulation, on the other
hand, is still rather precise under light 'Nembutal' anaesthesia
although this anaesthetic has changed the level towards which
the temperature is regulated. Consequently, it is unlikely that
adrenaline or noradrenaline exert these effects on the brain
stem through relays easily depressed by 'Nembutal'. Other regions
besides the reticular formation may also be influenced by adren-
aline. It is well known that adrenaline influences some reflex
activities at spinal levels (see e. g. SCHWEITZER and WRIGHT
1937; SKOGLUND 1952).

The maintenance of blood pressure during muscular work was
shown by U. S. VON EULER and LILJESTRAND (1946) to depend
strongly on baroreceptor reflexes, which include liberation of
catechols (FREEMAN and ROSENBLUETH 1931; LORD and HINTON
1945; HOLMGREN 1956). Catechols increase the sensitivity of the
baroreceptors (PALME 1943; LANDGREN 1952). Baroreceptor
reflexes primarily engage the splanchnic vascular bed and second-
arily the cutaneous vessels, both of which are also concerned with
thermoregulatory mechanisms.

BONVALLET et al. (1954) and DELL et al. (1954) have shown that
an increase in baroreceptor activity exerts a damping effect on
the activating systems of the brain stem. As has been discussed
above the baroreceptive activity during muscular work is high.
Partial blockage of the baroreflexes by a small dose of ergotamine
(ROTHLIN 1923; U. S. VON EULER and SCHMITERLÖW 1944) would
consequently increase shivering and activate cortical electrical
activity. That this was the case is illustrated in Fig. 7 B.

Respiratory and thermoregulatory needs may oppose each
other as is indicated by the effects of the various respiratory
gases on the threshold temperatures for shivering and on body
temperature. Either hypoxia or hypercapnea inhibited shivering
and thus had an effect opposite to that of adrenaline in spite of
the fact that either of these respiratory disturbances causes in-

creased liberation of catechols (RAAB 1943; BÜLBRING, BURN and DE ELIO 1948; KAINDL and U. S. VON EULER 1951; HÖKFELT 1951; HOUSSAY and RAPELA 1953). Oxygen on the other hand facilitated shivering markedly. These effects would tend to reduce the rate of rise of body temperature during muscular exercise, as oxygen saturation of arterial blood usually is decreased during heavy muscular work (ASMUSSEN and NIELSEN 1946). This is in accordance with the results of GELLHORN and JANUS (1936) that the body temperature of mice fell 3—4° C during anoxia. REIN (1935) obtained evidence indicating that the level of CO_2 could be of importance for the regulation of the metabolic rate presumably *via* central nervous structures.

KROGH (1935) has described a reflex whereby the sensitivity of the cold spots of the skin was increased, lowering the temperature threshold for cold sensation about 4° C. He assumed that the effect was mediated by sympathetic innervation of the cold receptors of the skin. It is possible that such an efferent control of sensitivity may have been involved in the change of hypothalamic temperature thresholds found in this work. The peripheral thermoreceptors of the skin may influence the hypothalamic temperature threshold considerably. In this respect there is great differences between different skin areas. The ears of the rabbit and also of the cat are of special importance for heat dissipation. The thermoreceptors of these skin areas were found not to be involved in governing thermoregulation. Conversely, the skin covering most of the body did not significantly participate in heat-dissipating vasomotor reflexes, but the thermoreceptors of these skin areas are certainly of importance for the control of body temperature. Heating or cooling these skin areas readily changes the hypothalamic temperature thresholds for thermoregulatory events. A close attachment of detector and effector functions which would give a positive feed-back arrangement is thus avoided (KÖNIG 1943; HENSEL 1952).

Summary.

1. In lightly anaesthetized cats and rabbits the threshold temperatures for several temperature regulating reactions, *i. e.*, onset and cessation of shivering and cutaneous vasoconstriction, were repeatedly determined when stimulating the animals in different ways.

2. It was found that various stimuli, such as nociceptive stimulation, pinna reflex, and electrical stimulation of the midbrain tegmentum which all activated the electroencephalogram and the gamma motor system, tended to raise the temperatures at which both heat-producing and heat-dissipating reflexes are brought into action with a rise in body temperature as a consequence.

3. Similar shifts of the temperature for thermo-balance were obtained by systemic infusion of adrenaline and noradrenaline. Changing the composition of the inspired gas was also found to influence thermoregulation.

4. Stimuli which inhibited the activity of the gamma motor system and evoked a synchronized cortical activity, e. g. stroking the back of the animal, lowered the threshold temperatures with a slight fall in body temperature as a result.

5. The results support the view that the body temperature is set at different levels in different states of activity.

References.

ALBERS, C., W. BRENDEL and W. USINGER, Pflügers Arch. ges. Physiol. 1956. *262*. 256.

ASMUSSEN, E. and O. BÖJE, Acta physiol. scand. 1945. *10*. 1.

ASMUSSEN, E. and M. NIELSEN, Acta physiol. scand. 1946. *12*. 171.

VAN DER BOGERT, F. and C. L. MORAVIC, J. Pediat. 1937. *10*. 466.

BONVALLET, M., P. DELL and G. HIEBEL, EEG Clin. Neurophysiol. 1954. *6*. 119.

BRENDEL, W., E. KOPPERMANN and R. THAUER, Pflügers Arch. ges. Physiol. 1954. *259*. 357.

BÜLBRING, E., J. H. BURN and F. J. DE ELIO, J. Physiol. 1948. *107*. 222.

CANNON, W. B., The wisdom of the body. New York, Norton & Co. 1932.

CANNON, W. B., A. QUERIDO, S. W. BRITTON and E. M. BRIGHT, Amer. J. Physiol. 1927. *79*. 466.

DELL, P., M. BONVALLET and A. HUGELIN, EEG Clin. Neurophysiol. 1954. *6*. 599.

EKSTRÖM, A., Acta physiol. scand. 1951. *22*. 345.

VON EULER, C. and U. SÖDERBERG, EEG Clin. Neurophysiol. 1957. *9*. 391.

von Euler, U. S., Noradrenaline. Springfield, Thomas 1956.

von Euler, U. S. and S. Hellner, Acta physiol. scand. 1952. 26. 183.

von Euler, U. S., S. Hellner-Björkman and I. Orwén, Acta physiol. scand. 1955. 33. Suppl. 118.

von Euler, U. S. and G. Liljestrand, Acta physiol. scand. 1946. 12. 298.

von Euler, U. S., G. Liljestrand and Y. Zotterman, Skand. Arch. Physiol. 1939. 83. 132.

von Euler, U. S. and C. G. Schmiterlöw, Acta physiol. scand. 1944. 8. 122.

Folkow, B., G. Ström and B. Uvnäs, Acta physiol. scand. 1949. 17. 317.

Freeman, N. E. and A. Rosenblueth, Amer. J. Physiol. 1931. 98. 454.

Gellhorn, E. and A. Janus, Amer. J. Physiol. 1936. 116. 327.

Granit, R., Receptors and sensory perception. New Haven, Yale University Press 1955.

Granit, R. and B. Kaada, Acta physiol. scand. 1952. 27. 130.

Hensel, H., Ergebn. Physiol. 1952. 47. 166.

Heymans, C., J. J. Bouckaert and L. Dautrebande, Arch. int. Pharmacodyn. 1931. 40. 54.

Hökfelt, B., Acta physiol. scand. 1951. 25. Suppl. 92.

Holmgren, A., Scand. J. clin. Lab. Invest. 1956. 8. Suppl. 24.

Holz, P., K. Credner and G. Kroneberg, Arch. exp. Path. Pharmak. 1947. 204. 228.

Houssay, B. A. and C. E. Rapela, Arch. exp. Path. Pharmak. 1953. 219. 156.

Kaindl, F. and U. S. von Euler, Amer. J. Physiol. 1951. 166. 284.

Kärki, N. T., Acta physiol. scand. 1956. 39. Suppl. 132.

Kleitman, N., Science 1945. 101. 507.

König, F. H., Pflügers Arch. ges. Physiol. 1943. 246. 693.

Krogh, A., Skand. Arch. Physiol. 1935. 71. 1.

Landgren, S., Acta physiol. scand. 1952. 26. 35.

Lord, J. W. and J. W. Hinton, J. A. M. A. 1945. 129. 1156.

Lusk, G. and J. A. Riche, Arch. Int. Med. 1914. 13. 673.

Nielsen, M., Skand. Arch. Physiol. 1938. 79. 193.

Palme, F., Z. ges. exp. Med. 1943. 113. 514.

Raab, W., J. Aviat. Med. 1943. 14. 284.

Rein, H., Nachr. v. d. Gesellsch. d. Wissensch. zu Göttingen (Biologie) 1935. 2. 229.

Rothballer, A. B., EEG Clin. Neurophysiol. 1956. 8. 603.

Rothlin, E., Arch. int. Pharmacodyn. 1923. 27. 459.

Schweitzer, A. and S. Wright, J. Physiol. 1937. 88. 476.

Skoglund, C. R., Cold Spr. Harb. Symp. quant. Biol. 1952. 17. 233.

Cortical Blood Flow Related to EEG Patterns Evoked by Stimulation of the Brain Stem.

By

DAVID H. INGVAR and ULF SÖDERBERG.

In recent years the influence of the brain stem upon the 'cortical excitatory state' (JASPER 1936) has been studied intensively by means of electrical stimulation of the reticular activating system (MORUZZI and MAGOUN 1949). In the non-anesthetized animal at rest, such stimulation as a rule gives rise to a general change of the EEG, characterized by a long-lasting transition from a more or less pronounced high voltage low frequency pattern (with or without spindles) into one containing higher frequencies of lower voltage. This response has been described under a number of terms such as 'desynchronization' of the EEG, 'electrographic arousal reaction', 'cortical activation', 'blocking reaction', etc. (ADRIAN and MATTHEWS 1934, MURPHY and GELLHORN 1945, JASPER and DROOGLEEVER-FORTUYN 1947, MORUZZI and MAGOUN 1949, cf. BONVALLET, DELL and HIEBEL 1954). It is, however, apparent that this response comprises a number of different EEG patterns amongst which several differ widely from the usual type described above. Thus, MORUZZI and MAGOUN (1949) observed that reticular stimulation sometimes gave an extreme flattening of the record with blocking of barbiturate spindles, a finding similar to the one of MORISON and DEMPSEY (1942). Brain stem stimulation has also been observed to induce a

pattern of synchrony with large slow waves instead of a desynchronization (MORISON, FINLEY and LOTHROP 1943, INGVAR 1955a). It should also be mentioned that HESS, KOELLA and AKERT (1953) succeeded in reproducing behavioural signs of sleep as well as the typical EEG pattern of sleep by stimulation of intralaminar thalamic areas.

The significance of the variations in the EEG response after brain stem stimulation has been poorly understood. Deviations from the most common 'activation' pattern have been explained as being due to deep anesthesia, or to a general deterioration of central nervous functions by the experimental conditions applied.

In a study of the effects of electrical stimulation of the brain stem upon the EEG we have recorded the cortical blood flow by a new, sensitive and continuous method (INGVAR and SÖDERBERG 1956a, b, 1958 INGVAR 1957). We have then observed that only an EEG response characterized by desynchronization with marked increase of frequency is accompanied by an increase of the cortical blood flow as described by INGVAR (1955b, 1957) and by INGVAR and SÖDERBERG (1956b). Other EEG responses to brain stem stimulation, including the above described 'flattening', were either not accompanied by any change at all, or else by a decrease of the cortical blood flow.

Methods.

Cats under 'Nembutal' (40 mg/kg intraperitoneally) or of the *encéphale isolé*-type (maintained by artificial respiration) were used.

The cortical blood flow was measured according to the method of INGVAR and SÖDERBERG (1956a, b, 1958, INGVAR 1957) by which the outflow from the cannulated superior sagittal sinus is recorded with an electric drop counter after elimination of the anastomoses to the diploic veins by a longitudinal craniotomy. In order to restore normal intracranial pressure, the bony defect was covered with dental acrylate cement (Svedon, Svedia Dental Industry AB, Enköping, Sweden) molded watertight around the sinus cannula and the stimulating and recording electrodes. The free end of the cannula was kept in a fixed position in relation to the sinus to provide constant pressure (generally slightly negative) throughout the experiments. The blood was returned to the animal by intravenous drop infusion at a rate equal to the outflow.

The cat's temperature was carefully kept constant by heating devices.

The arterial blood pressure in the femoral artery was registered by an electromanometer (ELEMA, Solna, Sweden).

EEG was recorded in bipolar leads by symmetrically placed silver ball electrodes over rostral and parietal parts of the cortex. The signals were fed to differentially coupled amplifiers which operated a two beam oscilloscope, or a 6-channel electroencephalograph, Offner, type A, or Grass, Modell III. In some experiments frequency analysis of the EEG was carried out by means of the optical method developed by KRAKAU (1951, 1953).

Needle electrodes (insulated to their tips) for electrical stimulation were orientated stereotaxically for an approach of supra- or infra-tentorial parts of the brain stem. Stimulus parameters (30—250 pulses/sec., duration 0.5—3 msec.) were individually variable. The stimulated points were checked histologically.

Results.

The effects to be described were obtained from electrical stimulation of both the bulbar and the meso-diencephalic reticular formation in anesthetized as well as unanesthetized (*encéphale isolé*) preparations. Different effects were occasionally observed to appear after stimulation of the same point in the same experiment, when paramenters of stimulation were changed. In other experiments the different effects could only be elicited from different points. A systematical mapping of the brain stem was not carried out.

In most cases, stimulation of the brain stem resulted in the well-known desynchronization of the EEG, commonly called the 'electrographic arousal reaction' which is characterized by a marked increase in frequency (MORUZZI and MAGOUN 1949). Normally in unanesthetized preparations this effect outlasted the stimulation period by several minutes. Parallel with the 'arousal reaction' of the EEG ran an increase of the cortical blood flow which lasted until the very moment when the synchronized pattern ('spindles') reappeared in the EEG (INGVAR 1955b, INGVAR and SÖDERBERG 1956a, b). As seen in Fig. 1 from an *encéphale isolé*-preparation (transection of the spinal cord at C1), the increase in the cortical flow cannot be explained by changes in systemic blood pressure, but must be due to a reduction of the cortical vas-
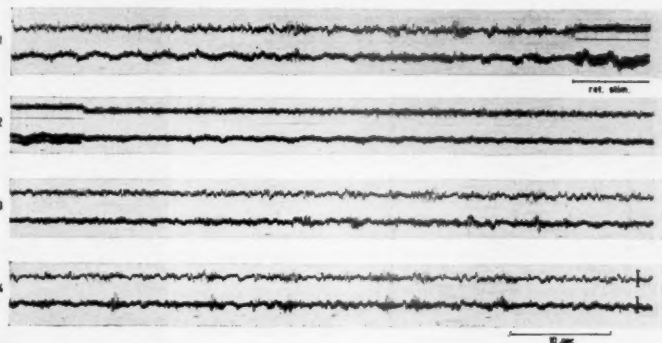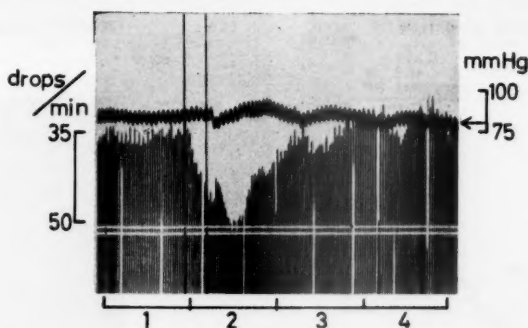
Fig. 1. Cat, *encéphale isolé* preparation. *Upper diagram:* Blood pressure and cortical blood flow. The light beams were interrupted every 30 sec. Height of vertical lines in blood flow record is proportional to time interval between consecutive blood drops from the cannula inserted into the superior sagittal sinus. Thus, a curve joining upper ends of vertical lines is inversely proportional to cerebral blood flow, cf. calibration to the left. *Lower diagram:* Four consecutive tracings of EEG from left cruciate and parietal areas, recorded on a rapidly running film. Time relation between upper and lower diagram demonstrated below blood flow record. Stimulation of mesencephalic reticular formation (A3, LO-1, H-2; 4 V, 200 c.p.s.) induces a marked 'arousal reaction' with a concomitant increase of cerebral blood flow although systemic blood pressure remains uninfluenced. When the electrical activity returns to the state before stimulation period blood flow also returns to the initial level.

cular resistance. (It should be mentioned that in a preparation with intact spinal cord the same stimulation would have elicited a very marked rise of blood pressure (INGVAR 1957).) The amount of increase in the cortical blood flow usually correlated well with duration and intensity of the arousal reaction of the EEG as well as, in time, with the changed frequency distribution in the ana-
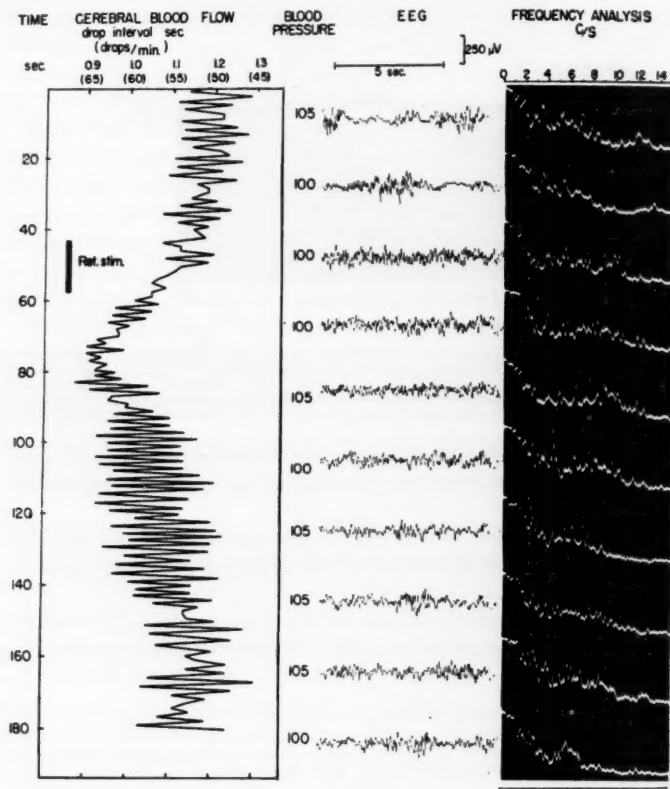
Fig. 2. Cat. *Encéphale isolé* preparation. Vagi sectioned. From left to right: Records of cortical blood flow (plotted from measurements of intervals between drops), blood pressure (femoral artery), samples of EEG (from the middle suprasylvian gyrus) and frequency analysis of the same samples *ad modum* KRAKAU (1951, 1953). The vertical bar on the left side of the figure indicates electrical stimulation of the meso-diencephalic reticular formation (250 c.p.s. duration 1 msec). The stimulation gives rise to an 'arousal reaction' of the EEG which is accompanied by an increase of the cortical blood flow in spite of the fact that the blood pressure remains uninfluenced. Spindles reappear in the EEG when the flow record returns to its original level. Frequency analysis (only including frequencies of up to 14 c.p.s) demonstrates a shift of the lower frequencies to higher ones during the arousal period. This shift gradually disappears and finally a frequency profile similar to the original one is obtained.
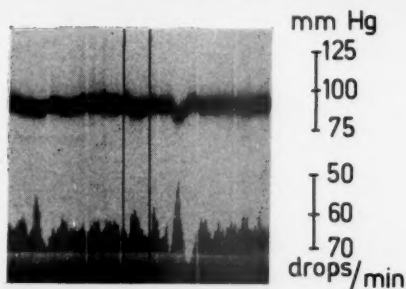
Fig. 3. Cat, light 'Nembutal' anesthesia. *Upper record:* Systemic blood pressure and cortical blood flow as in Fig. 1. Record interrupted every 30 sec. *Lower record:* Consecutive tracings of EEG from left parietal cortex. Electrical stimulation of medulla oblongata at the obex level three mm below the IV. ventricle (4 V, 32 c.p.s.) between two vertical lines in upper part and during horizontal bar in the middle of the lower record, elicits a marked reduction of the fast waves of the EEG throughout the period of stimulation. Cortical blood flow and arterial blood pressure are not affected.

lyzed EEG (Fig. 2). Thus, a short lasting 'arousal reaction' was only accompanied by a small change of blood flow.

A less frequent type of effect elicited from stimulation of the brain stem was characterized by very extreme reduction of the amplitude of the EEG with periods of complete flattening of the tracings occasionally interrupted by slow waves (Fig. 3). The example given in Fig. 3 represents an effect obtained from stimulation at the obex level of the brain stem. This type of response was, in fact, most often elicited from the bulb. In this experiment the 'flattening reaction' of the EEG only lasted during the period of stimulation while in other cases such as in Fig. 4 (mesencephalic stimulation) the 'flattening' of the EEG outlasted the period of stimulation for half a minute.

In contrast to earlier observations (for references see BOVET and LONGO 1956) the 'flattening reaction' was observed in both
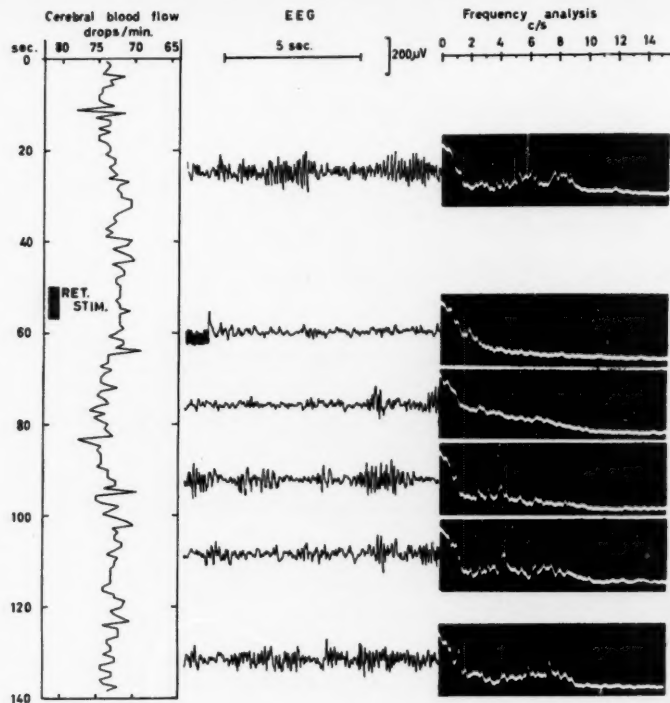
Fig. 4. *Encéphale isolé* preparation. Vagi and cervical sympathetic nerves sectioned. Records of cortical blood flow and EEG as in Fig. 2. The vertical bar on the left side indicates electrical stimulation of the meso-diencephalic reticular formation (250 pulses per sec. of 1 msec. duration). There is a marked flattening of the record after the stimulation outlasting the latter for a period of about 30 sec. Simultaneously the analyzed EEG demonstrates a general decrease of the frequency profile. Note the absence of any reaction of the cortical blood flow. The flow record shows a slight variation due to variations in the artifical respiration. The blood pressure varied between 90 and 95 mm Hg during the period illustrated.

anesthetized and unanesthetized preparations (*encéphale isolé*) but was more frequently observed in the anaesthetized animals. It was never accompanied by behavioural 'arousal'. There were no indications that the 'flattening reaction' pertained to any special cortical area.

Records of the cortical blood flow during a 'flattening reaction' demonstrated that it usually did not change significantly. Con-
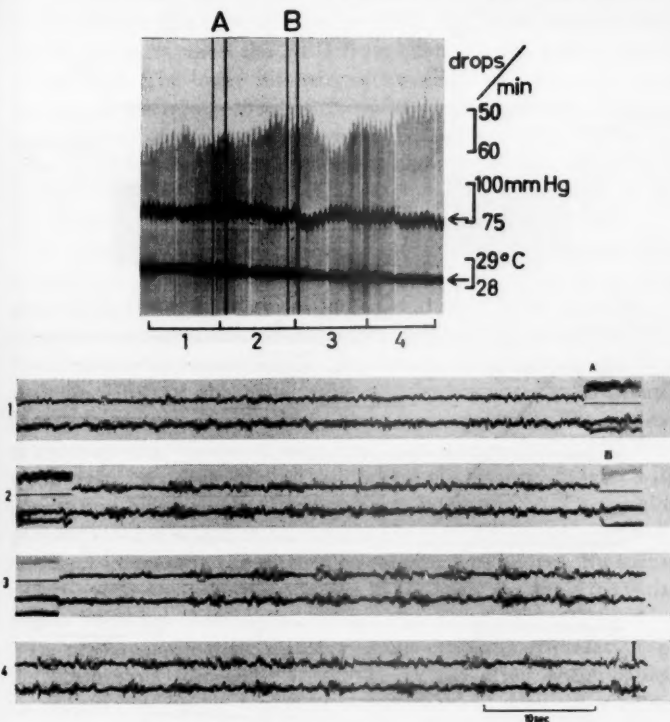
Fig. 5. Cat. *encéphale isolé. Upper diagram,* from above: Cortical blood flow as in Fig. 1, blood pressure and ear skin temperature. Record interrupted every 30 sec. *Lower diagram:* Consecutive tracings of EEG from left cruciate and parietal areas, as in Fig. 1 indicated by figures below upper diagram. Electrical stimulation of mesencephalic reticular formation 6 V (*A*) and 10 V (*B*), 200 c.p.s. have small or no immediate effects but are followed by a state of activity characterized by spindle configurations in the EEG. Cortical blood flow is uninfluenced.

stant flow, as well as constant systemic blood pressure were, in fact, the most common finding.

In confirmation of the results of HESS, KOELLA and AKERT (1953) it was also observed in several experiments that electrical stimulation of the brain stem gave rise to a sleep pattern in the EEG with typical spindle activity at frequencies of 8—12 c.p.s. An example of such a reaction is seen in Fig. 5 in which the mesencephalic reticular formation was stimulated. The figure demonstrates that no change of cortical blood flow and systemic pressure
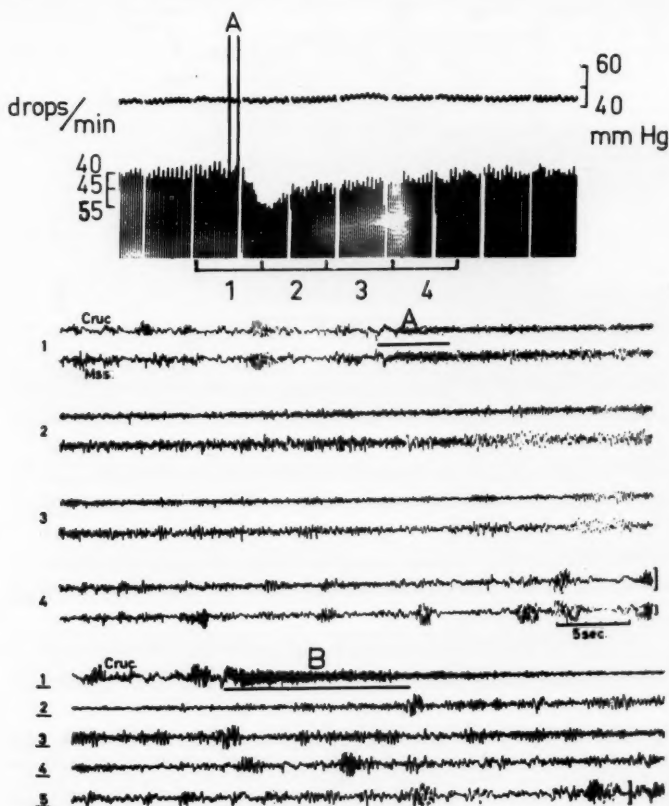
Fig. 6. Cat. *Encéphale isolé* preparation. Vagi, sympathetic nerves and sinus nerves sectioned bilaterally. Records of blood pressure and cortical blood flow and EEG as in Fig. 1. At *A* the mesencephalic reticular formation was stimulated electrically (250 pulses per sec. of 1 msec. duration). There is initially an 'arousal reaction' on the EEG which later is followed by a period of slow waves (about 4—5 per sec. starting at end of sample 2) before the EEG pattern assumes its original form with spindles (sample 4). This sequence is better seen in *B* (only EEG trace shown) in which the stimulation intensity was twice that in *A*. The period of cortical activation is shorter here and the slow waves more pronounced and of a lower frequency (about 3—4 per sec.). Note that the slow waves appear when the main events in the cortical circulation have surpassed. The blood pressure in this experiment was very low throughout and it remained uninfluenced by the brain stem stimulations.

occurred with this type of effect on EEG. Fig. 5 also demonstrates different effects upon the EEG from changing the parameters of stimulation. The lower intensity elicits sleep spindles only (even during the period of stimulation) while the stronger one induces a short period of 'arousal' followed by a pattern with spindling, even more marked than in the first case. Successive stimulation in this experiment produced, in fact, an augmentation of the spindle response of the EEG.

On some occasions electrical stimulation of the brain stem elicited a high voltage slow wave pattern. Such effects were often seen when the systemic circulatory conditions were impaired, or when the stimulus elicited an acute systemic circulatory failure. Often these reactions were preceded by a brief 'arousal reaction' with an EEG of the usual type which, however, subsequently changed into the high voltage slow wave pattern. The cortical circulation did not show any specific changes during the slow wave response, but diminished when circulatory failure with decrease of blood pressure was elicited. When the brief activation period preceded a slow wave response in the EEG, there was a con- comitant increase in the cortical circulation (see above). No sooner did the slow waves appear in the EEG than the main vascular events subsided (Fig. 6).

In preparations with intact nervous system, stimulation of the brain stem (especially in the bulb) was often accompanied by changes in muscle tone and respiration and sometimes apnea appeared. No correlation could, however, be found between these effects and the various reactions seen in the EEG and cortical blood flow, as described above.

In a few instances cerebral vasoconstriction as well as slight cerebral vasodilatation were elicited from electrical stimulation of points in the brain stem without any change in the EEG (cf. INGVAR and SÖDERBERG 1958).

Discussion.

In view of the observations reported on cortical blood flow during 'arousal reactions' it seems justified to differentiate one specific type of brain stem influence upon the electrical activity of the cortex from the others. This type is accompanied by a reduc- tion of the cerebral vascular resistance (Figs. 1 and 2) (cf. INGVAR

1955b, 1957, INGVAR and SÖDERBERG 1956b) whereas the other reactions described (Figs. 3 to 6), as a rule, are not followed by any detectable changes in cerebral circulation. This suggests that the term 'activation' had better be restricted to the first-mentioned type (cf. BONVALLET et al. 1954, p. 119) while the one illustrated in Figs. 3 and 4 might be called 'depression' or 'flattening' reaction. In the absence of information about neuronal behaviour at the unit level in the different states, it does not seem appropriate to apply the term 'inhibition' to the latter.

It should also be emphasized that the 'flattening reaction' obtained here, appears to be a physiological response. It does not imply reduction of the cortical circulation such as would follow from circulatory failure or an acute increase of the intracranial pressure. In such cases other varieties of EEG depressions are seen. Nor are there any reasons to assume that local vasoconstrictions account for the 'flattening' response, since it was often recorded simultaneously from several different cortical areas.

MORUZZI and MAGOUN (1949) and BOVET and LONGO (1956) have attributed the 'flattening reaction' of the EEG following brain stem stimulation to factors such as deep anaesthesia and/or a general deterioration of the preparation. Since the activation type and the flattening type could often be elicited in the same preparation, from the same point by merely altering the parameters of stimulation, as well as with or without anaesthesia (encéphale isolé), we are inclined to believe that both types of EEG effects represent physiological events, which may be differentiated by their respective cortical circulatory responses.

The EEG-pattern of the 'flattening reaction' differs clearly from synchronizing effects upon the EEG induced by brain stem stimulation of the kind described by MORISON, FINLEY and LOTHROP (1943) and also observed by INGVAR (1955 a). In these cases the EEG-response was characterized by a long-lasting period of high voltage slow waves (Fig. 6). Our findings support the notion that this type of EEG-response implies a failure of the cortical circulatory homeostasis in the face of the increased metabolic demands set up by the brain in response to the stimulation (INGVAR 1957). The state of increased cortical blood flow during the 'arousal response' is probably a sign of increased metabolic activity reflected in increased cortical oxygen consumption (ENGBERG et al. (1958).

BONVALLET et al. (1954) have reported that distension of the

wall of the carotid sinus has a synchronizing effect upon the EEG. Whether electrical stimulation of the brain stem, as in the present work, involves central relays of the sinus mechanism has not been studied.

Another type of EEG-response differing from the 'flattening reaction' is the 'sleep pattern' which may be produced both by electrical stimulation of intralaminar reticular nuclei of the thalamus (HESS, KOELLA and AKERT 1953), as well as of more caudal reticular parts of the brain stem. Our limited experience of this kind of response does not indicate the presence of any cortical vasomotor phenomena. It is obvious, however, that an experimental analysis of cerebral vasomotor phenomena in physiological sleep (SOKOLOFF 1956) involves many difficulties and that this problem therefore must await further study.

As exemplified in Fig. 5, transitional states between a response with sleep spindles and a high frequency 'arousal' could often be elicited by varying the intensity of stimulation. It was furthermore a frequent finding that a well developed 'arousal' response was followed by a more intense spindling than before stimulation (Fig. 5 and, to some extent, Fig. 1).

It should be emphasized that a distinct electrographic difference exists between the 'sleep response' and the high voltage slow wave pattern (Fig. 6.) The latter was seen much less frequently and — as pointed out above — often occurred during circulatory failure. It is also of interest that brain edema was sometimes seen to develop rapidly after a high voltage slow wave response. Edema was never seen after a sleep response, another observation indicating the different nature of the two phenomena.

Summary.

1. The cortical blood flow was recorded in lightly anesthetized or unanesthetized (*encéphale isolé*) cat preparations in which different EEG effects were elicited by electrical stimulation of the brain stem.

2. The regular EEG 'arousal reaction' ('cortical activation') was followed by a marked increase of the cortical blood flow which was due to a reduction of the cortical vascular resistance (INGVAR 1955b, INGVAR and SÖDERBERG 1956b).

3. From this was distinguished another type of EEG response,

the 'flattening reaction', characterized by reduction of amplitude with only occasional slow waves, differing significantly from the other by the fact that no cortical vasomotor changes were found to accompany it. Respiratory and systemic circulatory changes, as well as effects upon the muscle tone concomitant to the brain stem stimulation were not related to this response. It also seems unlikely that local vasoconstrictions could explain it. For these reasons it is set off as a specific reaction.

4. It was confirmed that 'sleep spindles' could be elicited by electrical stimulation of the brain stem. No cortical vasomotor changes could be recorded during such responses. Physiological sleep was not studied.

5. A fourth type of response was characterized by generalized high voltage slow waves. It often occurred during systemic circulatory failure and possibly had some relation to a defective cortical circulatory homeostasis and to the development of brain edema, seen in such cases.

References.

ADRIAN, E. D. and B. H. C. MATTHEWS, J. Physiol. 1934. *81*. 440.

BONVALLET, M., P. DELL and G. HIEBEL, Electroenceph. clin. Neurophysiol. 1954. *6*. 119.

BOVET, D. and V. G. LONGO, Abstr. Rev. XX. Int. Physiol. Congr. 1956. 306.

ENGBERG, I., D. H. INGVAR and B. SIESJÖ. 1958. To be published.

HESS, R., Jr., W. P. KOELLA and K. AKERT, Electroenceph. clin. Neurophysiol. 1953. *5*. 75.

INGVAR, D. H., Acta physiol. scand. 1955a. *33*. 137.

INGVAR, D. H., Acta physiol. scand. 1955b. *33*. 169.

INGVAR, D. H., Symp. on the Reticular Formation of the Brain. Henry Ford Hosp., Detroit. 1957. In press.

INGVAR, D. H. and U. SÖDERBERG, Nature 1956a. *177*. 339.

INGVAR, D. H. and U. SÖDERBERG, Electroenceph. clin. Neurophysiol. 1956b. *8*. 403.

INGVAR, D. H. and U. SÖDERBERG, 1958. To be published.

JASPER, H. H., Cold Spr. Harb. Symp. quant. Biol. 1936. *4*. 320.

JASPERR, H. H. and J. DROOGLEEVER-FORTUYN, Res. Publ. Ass. nerv. ment. Dis. 1947. *26*. 272.

KRAKAU, C. E. T., Electroenceph. clin. Neurophysiol. 1951. *3*. 97.

KRAKAU, C. E. T., Acta physiol. scand. 1953. *29*. 353.

MORISON, R. S. and E. W. DEMPSEY, Amer. J. Physiol. 1942. *135*. 281.

MORISON, R. S., K. H. FINLEY and G. N. LOTHROP, Amer. J. Physiol. 1943. *139*. 410.

MORUZZI, G. and H. W. MAGOUN, Electroenceph. clin. Neurophysiol. 1949. *1*. 455.

MURPHY, J. P. and E. GELLHORN, J. Neurophysiol. 1945. *8*. 339.

SOKOLOFF, P., Neurochemistry. S. R. KOREY and J. I. NURNBERGER, *Ed*. New York 1956.

Enzymic Systems Involving Adenosinephosphates in the Adrenaline and Noradrenaline Containing Granules of the Adrenal Medulla.

By

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The specific granules of the adrenal medullary cells store catechol amines (adrenaline + noradrenaline) and ATP[1] in nearly equivalent amounts (HILLARP, HÖGBERG and NILSON 1955; FALCK, HILLARP and HÖGBERG 1956). This suggests that ATP plays an important role in the storage and release of the amines. Further support to this view was obtained from the finding that stimulation of the medulla in vivo causes a drop in the ATP that is proportional to the decrease in the catechol amines (CARLSSON and HILLARP 1956; CARLSSON, HILLARP and HÖKFELT 1957).

Contrary to a report made by BLASCHKO, BORN, D'IORIO and EADE (1956), it has been found that the intragranular ATP is rapidly broken down when the granules are damaged (HILLARP and FALCK 1956). In the present work this breakdown has been further studied and attempts have been made to elucidate what enzymic systems involving adenosinephosphates are present in the granules. The aim of the experiments has been to study the enzyme activities of the granules from such aspects only as may be of interest for problems concerning the storage and release of the amines and ATP. Thus, the enzymic reactions have not been treated from a kinetical point of view.

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[1] The following abbreviations are used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5-phosphate; TCA, trichloracetic acid; PCA, perchloric acid; P_0, inorganic P; P_5, acid-labile P.

Material and Methods.

Isolation of the medullary granules: For every experiment adrenal glands from 4 to 8 cows were used. The glands were removed from the body about 30 minutes after the killing of the animals and immediately cooled with ice. After 30 to 60 minutes such parts of the medulla that were free from cortical tissue were dissected out.

The medulla was homogenized in 0.3 M sucrose as described in a previous paper (HILLARP, LAGERSTEDT and NILSON 1953). Unbroken cells and nuclei were removed by centrifugation at $800 \times g$ for 6 min. The supernatant was carefully decanted and about 40 ml, containing gran- ules from about 10 g of medulla, were centrifuged in a 50 ml tube for 20 min. at 15,000 rpm (MSE superspeed '20' refrigerated centrifuge, angle head 8×50 ml). The supernatant was discarded and the looser layer above the more tightly packed bottom sediment was carefully removed by swirling with sucrose. The granules of the bottom sediment were washed twice with 40 ml of 0.3 M sucrose and each time the looser layer was removed. On account of the high density of the specific granules, this isolation procedure gives a granule fraction which is contaminated only to a minor degree by mitochondria and microsomes (HILLARP 1958). The isolated granules were suspended in a few ml of 0.3 M sucrose.

To prepare the water insoluble residue the granules were suspended in a large volume of water or 0.015 M KCl (or NaCl) and centrifuged for 45 min. at 20,000 rpm (angle head 8×10 ml). The sedimented residue was washed once or several times with water or the dilute salt solution and finally suspended in water or KCl. All operations were carried out at $0°$.

To prepare the soluble protein fraction the granules were suspended in 0.015 M KCl and immediately centrifuged for 60 min. at 20,000 rpm (angle head 8×10 ml). The supernatant was mixed with an equal volume of ethyl alcohol, precooled to $-20°C$ and brought to pH 4 by adding a small volume of M HAc-NaAc buffer (pH 4). After 15 min. at $-25°C$ the precipitate was quickly spun down and washed with a large volume of cold 50 per cent ethanol. Most of the alcohol in the sedimented pro- teins was removed by a stream of N_2, care being taken not to dry the proteins. The proteins were dissolved in a 0.2 M histidine buffer (pH 7.5).

Standard test system for ATPase and adenylate kinase: The incubations were carried out at $+30°C$ in a mixture, used by KIELLEY and KIELLEY (1951), containing enzyme, 0.05 M histidine-HCl (pH 7.5), 0.04 M KCl, 0.005 M $MgCl_2$, 0.005 M ATP pH 7.5 (or ADP) and water in a final vol- ume of 3 ml. The medium was equilibrated for 10 min. at $+30°C$ before the cold enzyme was added. The incubation was interrupted by cooling the test tubes and adding 0.23 ml of 71 per cent PCA. The tubes were kept at $-6°C$ for 30 min. and then centrifuged. The sediment was washed with 2 ml of cold 0.4 N PCA and the combined extracts were neutralized to about pH 7 by means of KOH at $0°C$. After several hours at $-6°C$ the precipitated perchlorate was spun down and washed with 1 ml of cold water. The extracts were combined, made to a volume of 10.0 ml with water and kept at $-25°C$ until ion exchange chromatography and P-determinations were performed.

When determinations of ATPase activities were made, the incubation was interrupted by cooling and adding an equal volume of a cold 10 per cent solution of TCA. The tubes were kept at —6°C for 30 min. and then centrifuged. P-determinations were immediately made on the clear supernatant. In these experiments the incubation time was generally 10 min. and the enzyme amount was adjusted to such a level that not more than 10 per cent of the acid-labile P was liberated. Under these conditions the liberation of inorganic P was directly proportional to the enzyme concentration.

In all experiments suitable controls were used.

Determination of P: Inorganic P (P_0) and acid-labile P(P_8) (hydrolysis in N HCl for 8 min. at $+100°C$) were determined by the FISKE-SUBBAROW procedure (see LEPAGE 1951).

Determination of catechol amines: The total amount of adrenaline + noradrenaline was determined colorimetrically according to EULER and HAMBERG (1949).

Determination of protein-N: The proteins were precipitated by TCA or PCA and protein-N was determined according to the biuret method of CLELAND and SLAER (1953).

Ion exchange chromatography: All extracts were made with PCA and the perchlorate was removed as described above. Whenever catechol amines were present in the extracts, the amines were removed by passing an aliquot through a cation exchange column (Dowex 50, 150—300 mesh, 30×8 mm) previously equilibrated with M NaAc-HAc buffer of pH 4 or 6. In this form the exchanger permits AMP to pass through quantitatively. In all other cases the neutralized extracts were used directly.

An aliquot of the extracts, containing about 3 μmoles adenosinephosphates, was diluted with water and adjusted to pH 8.5 with ammonium hydroxide. The sample was then passed through an anion exchange column (Dowex 21, chloride form, 200—400 mesh, 21×6 mm) previously extensively washed alternatively with N HCl and N NaOH, thus reducing the ultraviolet absorption background of the chromatogram. The adenosinephosphates were eluted according to COHN and CARTER (1950), using a modified system of COHN (1951), at the rate of 0.2 ml per min.: 6×11.4 ml of 0.01 M NH_4Cl, 0.003 M HCl, 0.02 M NaCl in 0.01 M HCl and 0.2 M NaCl in 0.01 M HCl, respectively. The ultraviolet absorption of the various fractions was read in a Beckman spectrophotometer, Model DU. To check that no degradation of the adenine nucleus of the incubated adenosinephosphates had occurred, complete spectra of the eluted compounds were frequently taken.

Substances used: ATP (disodium salt), ADP (monosodium salt) and AMP (Pabst Laboratories or Sigma Chemical Company) were neutralized to pH 7.5 with NaOH and kept at —25°C. As seen from Tables II—IV each of the adenosinephosphates was contaminated by small amounts of the other two phosphates. Salyrgan was obtained from A-B Leo, Hälsingborg, stibophen (Fuadin) from Winthrop Laboratories and p-chloromercuribenzoate (sodium crystalline) from Sigma Chemical Company.

Results.

I. Breakdown of the intragranular ATP at lysis of the medullary granules.

The main phosphate esters in the adrenal medullary granules are ATP, ADP[1] and AMP[1] (HILLARP, HÖGBERG and NILSON 1955; FALCK, HILLARP and HÖGBERG 1956). In the present work the contents of catechol amines (22—25 μmoles/mg N) and of adenosinephosphates (about 5, 0.6 and 0.2 μmoles, respectively, per mg N) are higher than found earlier. This is due to the fact that in this work a granule fraction has been used which consists of the most easily sedimentable granules having only a small contamination by other cell structures and by damaged granules (HILLARP 1958).

The ATP and ADP which constitute more than 95 per cent of the total acid-labile phosphate esters in the granules, show a high stability in intact granules suspended in 0.3 M sucrose, no appreciable breakdown being found after several days at + 4°C. They are, however, rapidly split if the granules are damaged, *e. g.* by lysis in hypotonic media (HILLARP and FALCK 1956). If granules are transferred to hypotonic sucrose, NaCl or KCl solutions (unbuffered or buffered with HAc-NaAc or histidine, pH 6—7.5) inorganic P is liberated and reaches asymptotically the value for acid-labile P of the intact granules. The acid-labile P disappears at the same rate, no P_s remaining at the end of the experiments. The liberated inorganic P thus originates from the acid-labile phosphate esters only. Determinations of the reaction products (Table I) show that ADP and AMP are formed at the same rate as ATP disappears and that the end products are mainly AMP and inorganic P.

The data speak in favour of the view that the dephosphorylation of ATP to AMP takes place in at least two steps. The first step, ATP → ADP, seems to be relatively insensitive to NaF, the second, ADP → AMP, appears to be more sensitive to NaF (Table I). The data are readily explainable if the granule fraction contains the two well-known mitochondrial enzymes ATPase and adenylate kinase (cf. KIELLEY and KIELLEY 1953). This has been tested by using granules washed free from the endogenous phosphate esters

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[1] As yet only identified by ion exchange chromatography, ultraviolet absorption and by determination of their phosphorus and ribose contents.

Table I.

Reaction products formed from the ATP of medullary granules at lysis of the granules in water. The concentration of NaF in experiment B was 0.03 M.

Exp.	Incubation	ATP μM		ADP μM		AMP μM		ATP + ADP + AMP μM	Inorganic P μM		Acid-labile P μM		Inorganic + acid-labile P μM
		Total	Changes	Total	Changes	Total	Changes	Total	Total	Changes	Total	Changes	Total
A	Control 0 min.	1.85		0.22		0.08		2.15	0.42		4.10		4.52
	0° 60 min.	0.74	−1.11	0.88	+0.66	0.52	+0.44	2.14	1.87	+1.45	2.60	−1.50	4.47
	+37° 60 min.	0.07	−1.78	0.23	+0.01	1.75	+1.67	2.05	4.20	+3.78	0.32	−3.78	4.52
B	Control 0 min.	2.15		0.24		0.10		2.49	0.58		4.77		5.35
	+37° 15 min.	0.32	−1.83	1.15	+0.90	0.91	+0.81	2.38	3.39	+2.81	1.84	−2.93	5.23
	NaF +37° 15 min.	0.45	−1.70	1.75	+1.51	0.36	+0.26	2.56	4.13	+3.55	1.16	−3.61	5.29
	+37° 30 min.	0.25	−1.90	0.63	+0.39	1.70	+1.60	2.58	3.32	+2.74	1.83	−2.94	5.15
	NaF +37° 30 min.	0.35	−1.80	1.10	+0.86	0.92	+0.82	2.37					
	+37° 60 min.	0.16	−1.99	0.27	+0.03	2.15	+2.05	2.58	4.80	+4.22	0.55	−4.22	5.35
C	Control 0 min.	1.85		0.22		0.13		2.20	0.64		4.00		4.64
	+37° 30 min.	0.14	−1.71	0.26	+0.04	1.65	+1.52	2.05	4.10	+3.44	0.48	−3.52	4.58

Table II.

Reaction products formed in the standard test system incubated at $+30°$ with the insoluble granule residue (about 0.5 mg protein N/ml). The granule residue used in experiment B had been kept for 7 days at $-25°$. The concentration of NaF in experiment A was 0.04 M. For the chromatography 1/5 of the incubation mixture was used.

Exp.	Substrate	Mg++	Min.	ATP µM Total	ATP µM Changes	ADP µM Total	ADP µM Changes	AMP µM Total	AMP µM Changes	ATP + ADP + AMP µM Total	Inorganic P µM Total	Inorganic P µM Changes	Acid-labile P µM Total	Acid-labile P µM Changes	Inorganic + acid-labile P µM Total
A	ATP	++	0	2.90		0.21		0.15		3.26	0.84		5.75		6.59
	»	−	30	0.35	−2.55	1.50	+1.29	1.10	+0.95	2.95	4.24	+3.40	2.22	−3.53	6.46
	»	−	30	0.32	−2.58	2.43	+2.22	0.18	+0.03	2.93	2.65	+1.81	3.87	−1.88	6.52
	» +NaF	+	30	1.20	−1.70	1.55	+1.34	0.66	+0.51	3.40	5.35	+4.51	1.16	−4.59	6.51
	» +NaF	+	60	0.14	−2.76	1.15	+0.94	1.65	+1.50	2.94					
	ADP	+	0	0.19		2.95		0.26		3.40	0.84		3.15		3.99
	»	+	30	0.21	+0.02	1.20	−1.75	1.50	+1.24	2.91	2.64	+1.80	1.30	−1.85	3.94
	AMP	+	0	0.00		0.13		3.45		3.58	0.77				
	»	+	30	0.00	0.00	0.24	+0.11	2.80	−0.65	3.05	0.97	+0.20			
B	ATP	++	0	2.65		0.17		0.12		2.94					
	» +Ca++	+	30	0.96	−1.69	1.50	+1.33	0.31	+0.19	2.77					
	»	−	30	1.20	−1.45	1.60	+1.43	0.20	+0.08	3.00					
C	ATP	++	0	2.80		0.18		0.15		3.13					
	»	−	30	0.10	−2.70	1.76	+1.58	0.93	+0.78	2.79					
	»	+	30	0.04	−2.76	2.51	+2.33	0.40	+0.25	2.95					
	»	−	30	0.12	−2.68	1.84	+1.66	0.87	+0.72	2.83					
	»	−	30	0.04	−2.76	2.44	+2.26	0.41	+0.26	2.89					

and catechol amines which complicate the experiments, and by using granules not contaminated by mitochondria.

II. Enzymatic activities of the insoluble stroma of the medullary granules.

There is evidence that the medullary granules have a membrane with semipermeable properties enclosing the stored catechol amines and a water soluble protein fraction constituting a large part of the proteins of the granules (HILLARP and NILSON, 1954). The existence of a membrane has been shown in electron microscopic studies but no organized structures have as yet been observed inside the membrane (LEVER 1955; SJÖSTRAND and WETZSTEIN 1956; ROBERTIS and FERREIRA 1957). At lysis of the granules in hypotonic media, the sympathomimetic amines, the adenosinephosphates and the soluble proteins are released and may easily be separated from an insoluble residue by centrifugation at 35,000 × g. In the most pure granule fractions as yet obtained by density gradient centrifugation, the insoluble residue contains only 22—23 per cent of the total granule proteins (HILLARP, 1958). It seems probable that this residue consists of the granule membranes, but as it is possible that other organized structures exist in the granules — although not demonstrated in the electron microscope — the insoluble parts are in the following called stroma.

The granule stroma and the soluble protein fraction were tested, separately as well as together, for their ATP-splitting activity. Fig. 1 shows that the dephosphorylating enzymes are localized to the stroma and that the soluble contents of the granules neither inhibit nor appreciably activate the stroma enzymes. The result is the same whether the lysis takes place in water or in hypotonic electrolyte solutions. The appearance of small amounts of inorganic P during incubation of the soluble fraction in the experiment shown in Fig. 1 is an inconstant phenomenon and of unknown significance.

The stroma gives AMP and inorganic P as final products when ATP or ADP are used as substrates (Table II). The same result is obtained even if the stroma is extensively washed in water or dilute electrolytes in order to remove the soluble proteins. With AMP as a substrate small amounts of inorganic P may appear and it is probable that there is a deaminase of low activity (Section V).

By differential centrifugation of mechanically disintegrated mitochondria it is possible to separate an ATPase, removing only
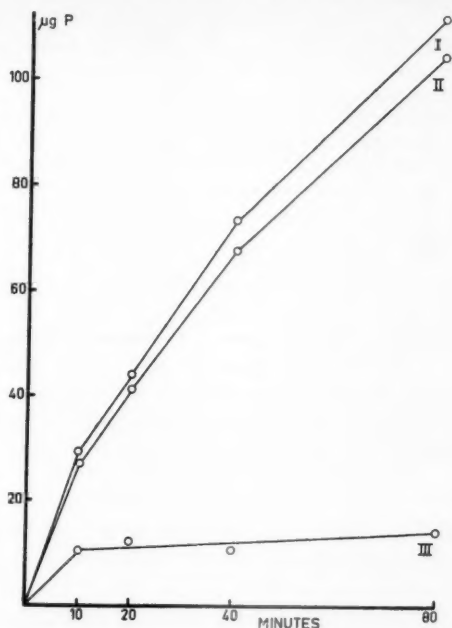
Fig. 1. Liberation of inorganic P per ml of the standard test system (ATP as substrate) and containing (I) the insoluble residue (0.10 mg protein N/ml) of medullary granules ruptured in water combined with their soluble content (0.12 mg protein N/ml), (II) the insoluble residue (0.10 mg N/ml), (III) the soluble content (0.12 mg N/ml).

the terminal phosphate group of ATP, from the mitochondrial adenylate kinase (KIELLEY and KIELLEY 1953). This method has been tried in the present work but no particles have been obtained showing a true ATPase activity only. The existence of a stroma ATPase splitting off only the terminal phosphate group can, however, be demonstrated by other means. Storage of washed stroma from lytically damaged granules at $-25°C$ was, in some instances, found to result in a more or less complete inactivation of the enzymes responsible for AMP-formation from ATP or ADP but to leave the reaction $ATP \rightarrow ADP + P$ relatively intact (Table II). Neither Mg^{++} nor Ca^{++} restored the stroma activity. Furthermore, it was found that freshly prepared and washed stroma exhibited only or mainly a true ATPase activity when incubated in a
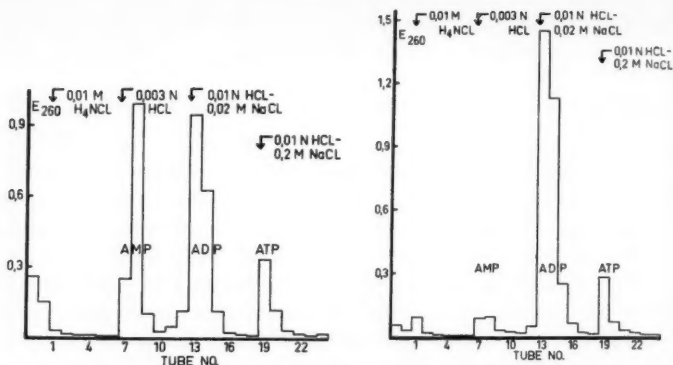
Fig. 2 and 3. Ion exchange chromatography of the reaction products formed in the standard test system (ATP as substrate) with (Fig. 2) or without Mg^{++} (Fig. 3) and containing the insoluble residue (0.48 mg protein N/ml) of medullary granules ruptured and washed in water. Incubation for 30 min. at $+30°$. For the chromatography 1/5 of the incubation mixture was used.

medium without Mg^{++} but otherwise of the usual composition (Fig. 3 and Table II). Addition of Mg^{++} restored the full enzymatic activity, resulting in AMP-formation (Fig. 2 and Table II). In several experiments, however, the removal of Mg^{++} has not resulted in an inactivity so complete as in the experiment shown in Fig. 3. No explanation has as yet been found for the observed variations in the enzymatic activity of different stroma preparations during incubation without Mg^{++}.

The transformation of ADP to AMP may be catalyzed by an ADPase or by an adenylate kinase. As myokinase is a relatively heat-stable protein in acid media (COLOWICK and KALCKAR 1943), the possibility of demonstrating the presence of an adenylate kinase in the granule stroma by destroying the ATPase at $+100°C$ was tested in the manner described in Table III. Heating of the stroma-suspensions almost completely destroyed their ATPase activity, which was tested by determinations of the inorganic P liberated during incubation with ATP. As seen from Table III, heating at neutral reaction to a large extent inactivated also the system giving AMP from ADP. In contrast to this, the stroma heated at pH 3—4, when incubated with ADP as a substrate, converted the diphosphate to approximately equimolar amounts of ATP and AMP.

There is thus an adenylate kinase as well as an ATPase in the

Table III.

Reaction products formed from ADP in the standard test system incubated at +30° with the insoluble granule residue (about 0.5 mg protein N/ml). The residue was washed twice in 0.015 M KCl and suspended in 0.15 M KCl. Aliquots of this suspension were heated in boiling water for 5 minutes. Before the heating HCl was added to some of the samples to adjust the pH to 3—4. These samples were neutralized with NaOH after the heating.

Exp.	Treatment	Incu-bation	ATP μM		ADP μM		AMP μM	
			Total	Changes	Total	Changes	Total	Changes
	Control	0 min.	0.10		2.60		0.25	
	Untreated	30 min.	0.16	+0.06	1.17	—1.43	1.40	+1.15
A	Heated with-							
	out acid	30 min.	0.26	+0.16	2.16	—0.44	0.44	+0.19
	Heated in acid	30 min.	0.78	+0.68	1.04	—1.56	0.96	+0.71
	Untreated	30 min.	0.12	+0.02	0.87	—1.73	1.61	+1.36
B	Heated in acid	30 min.	0.85	+0.75	1.15	—1.45	1.00	+0.75
	Heated in acid	60 min.	0.82	+0.72	0.89	—1.71	1.20	+0.95

insoluble stroma of the medullary granules. The presence of these two enzymes readily explains all observations concerning the products obtained with ATP or ADP as substrates and there is thus no need to postulate the existence of an ADPase. Furthermore, such an enzyme does not seem to have been demonstrated with any certainty in mammalian tissues, although a diphosphatase acting on nucleoside diphosphates other than ADP is present in mitochondria (GIBSON, AYENGAR and SANADI 1955; PLAUT 1955; GREGORY 1955). Some data speak against the presence of an ADPase in the granules. In the experiments where the adenylate kinase was inactivated at storage at —25°C, the ADPase, if present, must have been inactivated as well and whenever the adenylate kinase showed little or almost no activity without added Mg^{++}, the ADPase must have behaved in the same manner. This does not seem likely.

As the ATPase activity of the granule stroma and its changes with variations of pH — like that of mitochondria (KIELLEY and KIELLEY 1953) — are highly dependent on the ions present and their concentrations, the pH-activity relationship of the enzyme has been studied only quite superficially. In the standard test

system buffered by NaAc-HAc, boric acid-borate or tris(hydroxy-methyl)aminomethane buffers the maximal activity was found between pH 6 and 6.5, the activity rapidly decreasing at lower pH values but only slowly up to pH 8. A rather similar influence of pH on the dephosphorylation of the intragranular ATP was observed at lysis in hypotonic media containing Mg^{++}.

It may be questioned whether the ATPase and the adenylate kinase demonstrated in the insoluble residue of medullary granules are localized to the stroma of the specific catechol amine containing granules or belong to contaminating mitochondria which are known to contain these enzymes. In a separate work (HILLARP 1958) it has been found possible to isolate the specific granules practically free from mitochondria and microsomes by use of density gradient centrifugation. An analysis of the total and the specific ATPase activity of specific granules, with varying degrees of contamination by other cell structures, and of the various particle fractions that may be sedimented together with the specific granules, showed that the catechol amine containing granule fractions have the highest specific activities and that the main part of the total activity found in granule fractions, such as have been used in the present work, must belong to the specific granules. The specific activity of the purest fractions, having the highest content of catechol amines as yet found (about 35 μmoles/mg N), varied between 64 and 68 μmoles P liberated per hour and mg N (stroma proteins). Mitochondria from cow liver had a considerably lower — and mitochondria from rat liver a somewhat higher — specific activity when tested in the same manner.

It has as yet not been possible to get reliable data concerning the adenylate kinase activity owing to the technical difficulties involved in testing the small fractions obtained by density gradient centrifugation. It may be stated, however, that this enzyme is present in the stroma isolated from fractions of specific granules practically free from mitochondria and microsomes and that nothing has been found so far which speaks against the view that the catechol containing granules also contain an adenylate kinase (HILLARP 1958).

III. Enzymatic activities of the soluble proteins from the medullary granules.

The soluble protein fraction obtained at lysis of the medullary granules in hypotonic media contains a main component which

Table IV.

Reaction products formed from ADP in the standard test system incubated at +30° with the water soluble granule proteins (0.3—0.4 mg N/ml). The concentration of NaF in experiment C was 0.04 M and of CaCl₂ 0.005 M.

Exp.	Incubation		ATP μM		ADP μM		AMP μM		ATP+ADP+AMP μM
			Total	Changes	Total	Changes	Total	Changes	Total
A	0 min.......		0.08		2.72		0.12		2.92
	30 min.......	+ Mg^{++}	0.97	+0.89	1.09	—1.63	1.03	+0.91	3.09
B	30 min.......	+ Mg^{++}	1.16	+1.08	0.98	—1.74	0.95	+0.83	3.09
	60 min.......	+ Mg^{++}	1.10	+1.02	0.95	—1.77	0.93	+0.81	2.98
	30 min.......	— Mg^{++}	0.59	+0.51	2.06	—0.66	0.42	+0.30	3.07
C	30 min.......	+ Mg^{++}	0.95	+0.87	1.11	—1.61	0.93	+0.81	2.99
	30 min.......	— Mg^{+}	0.34	+0.26	2.31	—0.41	0.32	+0.20	2.97
	30 min. +Ca^{++}	— Mg^{++}	0.40	+0.32	2.19	—0.53	0.38	+0.26	2.97
	30 min. +NaF	+ Mg^{++}	0.48	+0.40	2.04	—0.68	0.47	+0.35	2.99

seems to be electrophoretically homogeneous and has an isoelectric point at about pH 4 (Högberg and Hillarp 1957). The fraction may conveniently be freed from catechol amines, adenosinephosphates and other substances by isoelectric precipitation (see Material and Methods).

A solution of these proteins in a histidine buffer of pH 7.5 does not show any ATPase activity and does not attack AMP, but has a high adenylate kinase activity (Fig. 4, Table IV). Starting with ADP as a substrate, the reaction catalyzed by this soluble adenylate kinase — like the myokinase reaction (Kalckar 1943) — comes to an equilibrium when about equimolar amounts of the three adenosinephosphates have been obtained. Like the adenylate kinase of the granule stroma, the soluble enzyme is activated by Mg^{++} (Figs 4 and 5) but not by Ca^{++} and is inhibited by NaF (Table IV).

In view of the possibility that the release of the catechol amines from the granules at secretion in vivo might involve a phosphorylation of the amines by the intragranular ATP, several attempts have been made to demonstrate such a reaction in vitro. If the whole water soluble content of the granules is incubated together
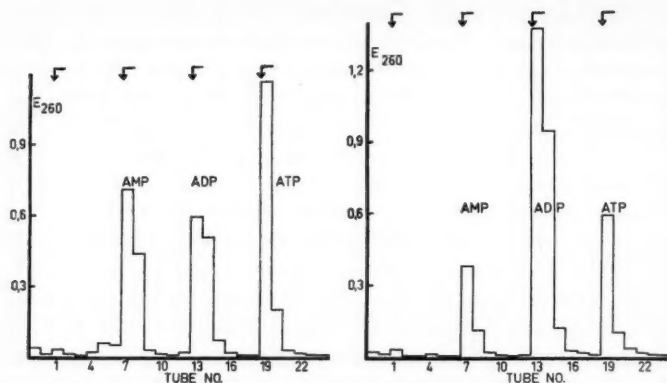
Fig. 4 and 5. Ion exchange chromatography of the reaction products formed in the standard test system (ADP as substrate) with (Fig. 4) or without Mg^{++} (Fig. 5) and containing the soluble granule proteins (0.45 mg N/ml). Incubation and chromatography as in Figs 2—3.

with added ATP and various cations, no changes of the adenosine-phosphates indicating an amine phosphorylation are found. If the test system is supplemented by the granule stroma, a phosphorylation cannot be studied in this way on account of the ATPase activity. Attempts to demonstrate the presence of phosphorylated catechol amines by the use of weak ion exchangers have failed, but probably the phosphate esters, if really formed, are too labile to be detected by this technique.

Concerning the question whether the soluble adenylate kinase is localized to the specific medullary granules or not, the same may be said as for the insoluble enzyme (Section II).

IV. ATPase and adenylate kinase activities of intact granules.[1]

When medullary granules, suspended in 0.3 M sucrose or in isosmotic KCl or NaCl, are kept at temperatures below $+20°C$ the intragranular ATP shows a remarkable stability in spite of the fact the ATPase of the granule is quite active at $0°$ (Table I). The stored ATP is in some unknown way protected from the ATPase. When incubated at $+30°$, the granules slowly release the catechol amines and parallel to this release the ATP is liberated and dephos-

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[1] The granules obtained by isolation in 0.3 M sucrose as described above (Material and Methods) are called "intact". This does not mean, however, that their properties are identical with those of the granules in the living cell.

Table V.

ATPase and adenylate kinase activities of intact medullary granules and of granules ruptured in water. In experiment A the granules were suspended in 0.3 M sucrose containing 0.005 M ATP (pH 7.5). In experiments B and C the granules were suspended in a medium containing 0.22 M sucrose, 0.04 M KCl, 0.005 M MgCl$_2$ and 0.005 M ADP (B) or AMP (C). In experiment B 1.67 μM ATP were added per ml of the suspencion containing intact granules. Incubation at +30° (A and B) or +25° (C).

Exp.	Incubation	ATP μM		ADP μM		AMP μM		ATP + ADP + AMP μM
		Total	Changes	Total	Changes	Total	Changes	Total
A	Control 0 min.	4.10		0.38		0.08		4.56
	Intact granules 20 min.	1.62	−2.48	2.50	+2.12	0.53	+0.45	4.65
	Ruptured granules 20 min.	1.04	−3.06	3.14	+2.76	0.43	+0.35	4.61
B	Control 0 min.	1.95		2.80		0.23		4.98
	Intact granules 15 min.	1.85	−0.10	2.30	−0.50	0.78	+0.55	4.93
	Intact granules 30 min.	1.55	−0.40	2.20	−0.60	1.08	+0.85	4.83
	Control 0 min.	0.95		2.85		0.25		4.05
	Ruptured granules 15 min.	0.92	−0.03	2.20	−0.65	0.90	+0.65	4.02
	Ruptured granules 30 min.	0.69	−0.26	2.10	−0.75	1.25	+1.00	4.04
C	Control 0 min.	0.95		0.18		3.05		4.18
	Intact granules 30 min.	0.90	−0.05	0.24	+0.06	3.00	−0.05	4.14

phorylated (or first dephosphorylated). In contrast to this, ATP and ADP added to the granule suspension are reladily attacked by the ATPase and the adenylate kinase, respectively.

In Table V the enzymatic activities of intact and of lytically damaged granules are compared. In experiment A, ATP was added to granule suspensions in 0.3 M sucrose without Mg^{++} or KCl. Although the ATPase reaction has almost reached the asymptotic

level, it is obvious that the activity of the intact granules is comparable to that of the granules destroyed by lysis. The same holds true for the adenylate kinase activity in experiment B, where ADP was substrate, the sucrose suspension was supplemented by KCl and Mg^{++} and where ATP was added to the intact granules to get the same concentration of free ATP in both systems. As the dismutation of ADP gives equimolar amounts of AMP and ATP, it is obvious from the data that the two systems had about the same ATPase activity as well. This activity is rather low, which may depend on the low substrate level and maybe on an inhibition by ADP. The ATPase of myosin (KALCKAR 1944) and of mitochondria (KIELLEY and KIELLEY 1953; POTTER, SIEKEVITZ and SIMONSON 1953) is inhibited by ADP.

The intact granules used in the experiments described above are to some extent contaminated by the insoluble residue of damaged granules and by mitochondria (HILLARP 1958). It is quite unlikely, however, that these structures could account for the activities found, unless the soluble content released from the granules at lysis contains some strong enzyme inhibitor. It has been shown that this is not the case (Fig. 1). Many ions and substances in low concentrations have been found to damage the granule membrane giving a release of the soluble content. However, the adenosinephosphates and Mg^{++}, in the concentrations used above, neither activate nor inhibit the spontaneous release of catechol amines occurring at $+30°$. It therefore seems that the evidence speaks much in favour of the view that ATP and ADP readily penetrate from the outside to the enzyme sites in the granules.

As there is evidence (Section III) that the soluble proteins in the interior of the granules contain an adenylate kinase, it is o considerable interest to know if the intragranular ATP is stored in such a way that it may react with this enzyme. To test this possibility intact granules were incubated in sucrose supplemented with KCl, Mg^{++} and AMP. There were, however, no changes of the intragranular adenosinephosphates (Table V:C).

V. Enzymes attacking AMP.

When intact or lytically damaged granules were incubated at $+30°$ or $+37°C$ the total amounts of adenosinephosphates (added or endogenous) did not change (Tables I and V). The same holds true for the soluble granule proteins (Table IV). There was, however, a loss in several experiments where the granule stroma was

used (Table II). This disappearance, which was most pronounced with AMP as the substrate, could be demonstrated also by directly following the decrease of the ultraviolet absorption at 260 mμ. At ion exchange chromatography a new compound appeared just preceding the ADP fraction. The elution position of the compound (cf. DEUTSCH and NILSSON 1953) and its ultraviolet absorption strongly suggest that it is inosinemonophosphate. There may thus be a 5-adenylic acid deaminase in the preparations of granule stroma. However, rough calculations on the maximal amounts of inosinemonophosphate that might have been formed seem to show that a deamination cannot account for all of the AMP which disappeared. As small amounts of inorganic P were liberated when AMP was substrate, an AMPase may be assumed to be present in the preparations. No further studies on the enzymes attacking AMP have been made, however, on account of the difficulties in demonstrating that the enzymes really belong to the specific granules.

VI. Inhibition and activation of the granule enzymes.

As it may be possible that the stroma ATPase of the medullary granules in some way is linked to the mechanism for the release of the stored catechol amines (see Discussion), a search has been made for inhibitors that might be used in experiments with isolated granules.

Several metal ions give, when added to suspensions of intact granules in 0.15 M KCl at +30°C a very rapid release of the catechol amines. Some of the ions (10^{-3} M Al^{+++}, Cu^{++}, Fe^{+++}) strongly inhibited the dephosphorylation of the concomitantly liberated ATP, others did not (10^{-3} M Mn^{++}, Zn^{++}, Cd^{++}).

Myosin ATPase and many P-transferring enzymes seem to be thiol enzymes (cf. BARRON 1951). Of the typical —SH reagents only p-chloromercuribenzoic acid (PCMB) has been tested, and at a concentration of 10^{-3} M it showed a more than 50 per cent inhibition of the stroma ATPase activity determined in the standard test system. Unfortunately PCMB cannot be used to elucidate the role of this enzyme for the amine release from intact granules as it causes a rapid lysis of the granules. Salyrgan, which inhibits myosin ATPase (PORTZEHL 1952), showed about the same inactivation of stroma ATPase as PCMB but also destroys intact granules. Stibophen (10^{-3} M), another inhibitor of the muscle ATPase (PORTZEHL 1954; WEBER 1955), did not show any effect on the

stroma ATPase (standard test system). Of the agents commonly used to oxidize —SH groups, only ferricyanide (10^{-3} M) has been tested. It caused a rapid amine release from intact granules without any appreciable inhibition of ATP breakdown.

The posphatase inhibitors polyphloretine phosphate (50 μg/ml) and sodium borate (0.1 M) had no obvious effects on the ATP-splitting occurring at lysis of intact granules. NaF (0.01—0.03 M) was in a previous work (HILLARP and FALCK 1956) not found to inhibit the liberation of inorganic P to any appreciable degree when granules were transferred to a hypotonic HAc-NaAc buffer (pH 6). Since then several similar experiments have been made with the same result. From the single experiment recorded in Table II (A) it is obvious, however, that 0.04 M NaF in the standard test system at pH 7.5 may cause a considerable inhibition of the stroma ATPase. NaF in this concentration strongly depressed the adenylate kinase activity (Table IV : C).

It is well-known that in general the ATPases and other enzymic systems involving ATP are Mg^{++}-activated. This may be true for the stroma ATPase as well, even if it does not show in the experiments recorded in Table II (A and C), where the effect of Mg^{++} on the initial reaction velocities cannot be seen. That the adenylate kinase activity of the insoluble stroma as well as of the soluble proteins is activated by Mg^{++} is evident (Table II, A and C; Table IV, B and C).

In view of the pronounced activating effect of 2,4-dinitrophenol on the latent ATPase of mitochondria (HUNTER 1951; POTTER and RECKNAGEL 1951; LARDY and WELLMAN 1953), its effect on the medullary granules was tested. Intact granules were incubated at $+30°$ in a medium, made isotonic by means of sucrose, containing KCl, Mg^{++} and ATP. No obvious stimulation of the ATPase activity could be elicited by dinitrophenol (4×10^{-4} M). Neither did this substance cause a release of the catechol amines or any changes in the intragranular adenosinephosphates.

Discussion.

On account of the large amounts of ATP found in the specific granules of the adrenal medulla, it was suggested in a previous paper that ATP may have an important role in the storage and release of the catechol amines (FALCK, HILLARP and HÖGBERG 1956). The following findings strongly support this view:

1. Previous calculations of the molar ratio of catechol amines to ATP showed that this ratio could not be much above 4:1, which corresponds to equivalent amounts of acid and base. More exact determinations made on granule fractions of high purity (HILLARP 1958) are in good agreement with the theoretical value calculated on the basis of the average net ionic charge of ATP (ALBERTY, SMITH and BOCK 1951) and of adrenaline (LEWIS 1954) at neutral reaction. This might be a coincidence but its significance is strengthened by the finding that catechol amines and ATP (together with small amounts of ADP, AMP and inorganic phosphate) represent almost the whole ionic content of water extracts of granules isolated in sucrose (CARLSSON and HILLARP 1958).

2. When the catechol amines in the adrenal medulla are decreased by stimulation of the medulla in vivo, there is a disappearance of ATP from the granules proportional to the drop in amines (CARLSSON and HILLARP 1956; CARLSSON, HILLARP and HÖKFELT 1957).

3. Extensive studies (unpublished) on the properties of the isolated granules show that under all conditions used ATP is, on the one hand, always split or released when the catechol amines are liberated and that it, on the other hand, is stable so long as no amine release takes place.

It seems difficult to avoid the conclusion that catechol amines and ATP are stored in intimate connection to each other. Thus it may be useful to approach the problem of the storage and release of the amines from the ATP point of view. All granule enzymes involving the adenosinephosphates are consequently of special interest.

A great difficulty in studies on the enzymatic activities of the medullary granules is to get convincing evidence that an observed activity really belongs to the specific amine containing granules. There are good reasons to believe that an ATPase is localized to the granules (see Section B) but the evidence in this respect for the adenylate kinase is far from conclusive. Although it has been possible to demonstrate the presence of these enzymes in granule fractions practically free from mitochondria, it must be kept in mind that mitochondria, at least from liver, show a very high ADP transphosphorylase activity (BARKULIS and LEHNINGER 1951; KIELLEY and KIELLEY 1951; SIEKEVITZ and POTTER 1953). The adenylate kinase of mitochondria and that of the medullary granules are both activated by Mg^{++}, inhibited by NaF and

solubilized by water treatment (see also SIEKEVITZ and WATSON 1956). There is a difference, although of unknown significance, in that the liver enzyme has been reported not to be heat-stable in acid (KOTELNIKOVA 1949). In the following the adenylate kinase is discussed under the assumption that it is present in the specific granules; but the uncertainty of this assumption must be kept in mind, however.

The ATPase activity of the medullary granules is exclusively localized to the insoluble residue obtained at lysis of the granules and is not removed by repeated extractions using water or dilute electrolyte solutions. It is tempting to assume that the enzyme is situated in or on the granule membranes, which in all probability are a main part of the residue. Some support for this view is obtained from the observation that ATP readily seems to penetrate to the sites of enzyme activity when added to intact granules. However, it is not known how far ATP may penetrate into the granules and the fact that the ATPase activity is not removed by water or salt extractions does not necessarily mean that it is a membrane constituent of intact granules. The enzyme may exist in the interior of the granules and may be insoluble in the extraction solutions used. This possibility certainly must be considered, among others, in view of new studies on the erythrocyte ATPase (CAFFREY, TREMBLAY, GABRIO and HUENNEKENS 1956).

The presence of adenylate kinase activity both in the insoluble residue and in the water soluble content released at lysis of the medullary granules may mean that there are two different enzymes, a soluble enzyme within the granules and an enzyme bound to the membrane or some other structural element. The experiments do not exclude, however, that the activity of the residue is a result of a secondary adsorption of a soluble adenylate kinase or that an enzyme bound to the structural elements by weak forces is only partly solubilized at lysis. It is of interest that a part of the mitochondrial ADP transphosphorylase is relased and a part held to the membranes if mitochondria are treated with water (SIEKEVITZ and WATSON 1956) and that metabolic studies point to the existence of two sites of adenylate kinase activity in mitochondria (SIEKEVITZ and POTTER 1955).

ADP seems readily to reach a site of adenylate kinase activity from the outside of intact granules. On the other hand no transphosphorylation takes place between the intragranular ATP and AMP penetrating from the outside. This argues in favour of the

view that an ADP transphosphorylase is present in an outer zone separated from the ATP within an inner part of the granules.

The presence of a highly active ATPase in the medullary granules may suggest that this enzyme plays a role in the release (or storage) of the catechol amines. Only vague speculations can be made at present, however. The uncertainty is accentuated by the fact that there is no clear evidence against the possibility that ATP, together with the catechol amines, is secreted from the medulla. ATP has been shown to influence some autonomic effector systems in concentrations that well may be obtained in the blood by such a secretion. This possibility does not seem very likely, however, as long as no evidence at all exists in favour of it.

Under "resting" conditions the intragranular ATP is protected from the ATPase in some unknown way. It is tempting to speculate that a stimulation of the medullary cell changes the granules enabling the enzyme to attack ATP. In view of the close connection between the stored catechol amines and ATP, a dephosphorylation may well be assumed to set free amines which, together with the inorganic phosphate produced, may be released from the granules by diffusion or by active transport using energy derived from the ATP-splitting. The adenylate kinase may then act on the ADP produced at this splitting giving ATP and AMP and the latter may leave the granules together with an equivalent amount of amines. The only observation (unpublished) that may give some little support to this hypothesis is that isolated granules in certain media show a small, initial accumulation of inorganic phosphate just as an amine release is starting, indicating that a dephosphorylation of ATP precedes the release.

Summary.

1. The ATP that is stored in large amounts, together with the sympathomimetic amines, in the specific granules of the adrenal medulla is rapidly broken down to AMP when the granules are ruptured in hypotonic media. This breakdown is brought forth by a true ATP-ase, removing only the terminal phosphate group of ATP, and by an adenylate kinase.

2. There is good evidence that the ATPase activity is localized to the specific granules. The adenylate kinase activity probably belongs to these granules but the evidence is not conclusive.

3. The ATPase activity is exclusively localized to the water insolu-

ble residue obtained at lysis of the granules and probably to a main part consisting of granule membranes. This residue also shows an ADP transphosphorylase activity which is not removed by repeated washings and is heat-stable in acid. A soluble adenylate kinase is released from the granules at lysis in water or in dilute salt solutions. There is no further evidence, however, that there really are two different ADP transphosphorylases in the granules.

4. The adenylate kinase activity is activated by Mg^{++} and inhibited by NaF. The ATPase activity is inhibited by p-chloromercuribenzoate and salyrgan but not by stibophen and it is relatively insensitive to NaF at pH 6 where maximal activity is found. Under other conditions NaF may give appreciable inhibition.

5. ATP and ADP seem readily to penetrate to the sites of enzyme activity from the outside of intact granules. No transphosphorylation between the intragranular ATP and AMP penetrating from the outside could be demonstrated, however, and the stored ATP is in some way protected from the ATPase under "resting" conditions. It may thus be that the ATPase and the ADP transphosphorylase are present in an outer zone separated from the ATP stored within an inner part of the granules.

6. The intimate connections between the catechol amines and the ATP stored in the granules and a possible role of the ATPase in the amine release are discussed.

References.

ALBERTY, K. A., R. M. SMITH and R. M. BOCK, J. biol. Chem. 1951. *193*. 425.

BARKULIS, S. S. and A. L. LEHNINGER, J. biol. Chem. 1951. *190*. 339.

BARRON, G. E. S., in Advances in Enzymology (Ed.: F. F. NORD) *XI*. p. 201. New York 1951.

BLASCHKO, H., G. V. R. BORN, A. D'IORIO and N. R. EADE, Biochem. J. 1956. *62*. Part 2. 18 P.

CAFFREY, R. W., R. TREMBLAY, B. W. Gabrio and F. M. HUENNEKENS, J. biol. Chem. 1956. *223*. 1.

CARLSSON, A. and N.-Å. HILLARP, Acta physiol. scand. 1956. *37*. 235.

CARLSSON, A. and N.-Å. HILLARP, 1958 (to be published).

CARLSSON, A., N.-Å. HILLARP and B. HÖKFELT, J. biol. Chem. 1957. *227*. 243.

CLELAND, K. W. and E. C. SLATER, Biochem. J. 1953. *53*. 547.

COHN, W. E., J. cell. comp. Physiol. 1951. *38*. 21.

COHN, W. E. and C. E. CARTER, J. Amer. chem. Soc. 1950. 72. 4273.
COLOWICK, S. P. and H. M. KALCKAR, J. biol. Chem. 1943. 148. 117.
DEUTSCH, A. and R. NILSSON, Acta chem. scand. 1953. 7. 1288.
EULER, U. S. v. and U. HAMBERG, Acta physiol. scand. 1949. 19. 74.
FALCK, B., N.-Å. HILLARP and B. HÖGBERG, Acta physiol. scand. 1956. 36. 360.
GIBSON, D. M., P. AYENGAR and D. R. SANADI, Biochim. biophys. Acta 1955. 16. 536.
GREGORY, J. D., Fed. Proc. 1955. 14. 221.
HILLARP, N.-Å., 1958 (to be published).
HILLARP, N.-Å. and B. FALCK, Acta endocr. (Kbh.) 1956. 22. 95.
HILLARP. N.-Å., B. HÖGBERG and B. NILSON, Nature 1955. 176. 1032.
HILLARP, N.-Å., S. LAGERSTEDT and B. NILSON, Acta physiol. scand. 1953. 29. 251.
HILLARP, N.-Å. and B. NILSON, Acta physiol. scand. 1954. 31. Suppl. 113.
HUNTER, F. E., in McELROY, W. D. and B. GLASS, Phosphorus Metabolism 1. 297. Baltimore 1951.
HÖGBERG, B. and N.-Å. HILLARP, 1957 (unpublished).
KALCKAR, H. M., J. biol. Chem. 1943. 148. 127.
KALCKAR, H. M., J. biol. Chem. 1944. 153. 355.
KIELLEY, W. W. and R. K. KIELLEY, J. biol. Chem. 1951. 191. 485.
KIELLEY, W. W., J. biol. Chem. 1953. 200. 213.
KOTELNIKOVA, A. V., Biokhimija 1949. 14. 145.
LARDY, H. A. and H. WELLMAN, J. biol. Chem. 1953. 201. 357.
LEPAGE, G. A., in UMBREIT, W. W., R. H. BURRIS and J. F. STAUFFER, Manometric Techniques, 2nd edit. p. 185. Minneapolis 1951.
LEVER, J. D., Endocrinology 1955. 57. 621.
LEWIS, G. P., Brit. J. Pharmacol. 1954. 9. 488.
PLAUT, G. W. E., J. biol. Chem. 1955. 217. 235.
PORTZEHL, H., Z. Naturforsch. 1952. 7b. 1.
PORTZEHL, H. Biochim. biophys. Acta 1954. 14. 195.
POTTER, V. R. and R. D. RECKNAGEL, in McELROY, W. D. and B. GLASS, Phosphorus Metabolism, 1. 377. Baltimore 1951.
POTTER, V. R., Ph. SIEKEVITZ and H. C. SIMONSON, J. biol. Chem. 1953. 205. 893.
ROBERTIS, E. de and A. VAZ FERREIRA, Exp. Cell. Res. 1957. 12. 568.
SIEKEVITZ, P. and R. V. POTTER, J. biol. Chem. 1953. 200. 187.
SIEKEVITZ, P. and V. R. POTTER, J. biol. Chem. 1955. 215. 237.
SIEKEVITZ, P. and M. WATSON, J. Biophys. and Biochem. Cytol. 1956. 2. 653.
SJÖSTRAND, F. S. and R. WETZSTEIN, Experientia (Basel) 1956. 12. 196.
WEBER, H. H., Proc. 3rd Int. Congr. Biochem. Brussels 1955. p. 356.

Some Characteristics of the Renal Tubular Transport Mechanism for Histamine in the Hen.

By high

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Histamine is excreted by the tubules in the chicken (Lindahl and Sperber 1956). Our knowledge of this process is, however, very limited. In order to fill some of the gaps, further experiments on the tubular excretion of histamine are reported in this paper.

In some cases it has been shown that the pH of the urine is of decisive importance for the tubular excretion of a substance (*e. g.* KEMPTON 1939). It was therefore considered necessary to study the influence of variations in the urinary pH on the tubular excretion of histamine.

At least two relatively well defined excretory mechanisms exist in the renal tubules (for review of literature see SMITH 1951). In order to investigate whether the tubular excretion of histamine is connected to either of these, the influence of brom cresol green, priscoline, and cyanine # 863 on the excretion of histamine has been examined.

An attempt has also been made to determine the maximal transport capacity of the tubules for histamine.

Methods.

In the present investigation as in the previous one (LINDAHL and SPERBER 1956) we have used a modification of a method for investigating renal secretory phenomena in birds, developed by

one of us (SPERBER 1948). This method makes use of the circumstance that the renal portal system in the birds receives part of its blood supply from the legs. In consequence a substance injected into one of the legs passes the peritubular capillaries of the ipsilateral kidney before entering the general circulation. Provided the tubules possess a mechanism of secretion acting on this substance, at least part of the substance injected is transferred to the urine during this passage. The subsequent excretion of the substance will be equally distributed between the two kidneys. Thus the difference between the amounts excreted by the ipsilateral and the contralateral kidney, divided by the dose injected, may be taken as a measure of the efficiency of the tubular excretion with regard to the compound investigated. It is called the apparent tubular excretion fraction (ATEF).

White Leghorn hens were used. The separate collection of the urine formed by the two kidneys was made possible by the suturing at each ureteral opening of a polyethylene funnel, which was irrigated with 0.2 per cent sodium chloride. For further experimental details see LINDAHL and SPERBER 1956.

Test solutions containing phenol red in addition to histamine and, whenever wanted, priscoline and brom cresol green, were infused at a rate of 0.33 ml/min. into the renal portal circulation via a leg vein. Cyanine, when used, was injected separately into the same vein during the injection of histamine and phenol red.

Earlier experiments have shown that about 98 per cent of the excreted histamine appears within 10 minutes after the injection. The rapid cessation of excretion after an infusion period makes it possible to make a series of injections in the same animal within a short time. Thus an experiment on a single hen consisted of a series of injections comprising alternating control and test periods. Every injection was followed by a collection period of 15 minutes.

The phenol red concentrations were determined by measuring the light absorption at an alkaline pH at 558 mμ.

Histamine activity was assayed on guinea pig ileum. The samples were diluted from 5 to 100 times, avoiding thereby the effects of urinary components that influence the contractions of the gut. Phenol red, brom cresol green, priscoline, and cyanine did not influence the histamine assay in concentrations comparable to those occurring in the samples.

The pH of the urine was changed by hydrating the hens with sodium bicarbonate solution by crop tube. pH was measured
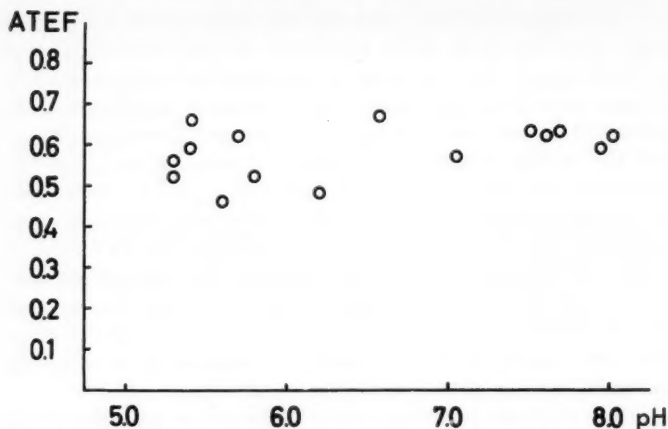
Fig. 1. The distribution of urine pH and the apparent tubular excretion fraction of histamine.

potentiometrically. No precautions were taken to avoid CO_2-losses, and the pH values given are to some degree influenced by this factor. Control experiments have, however, shown that the strong buffer effect of the uric acid makes this influence very slight.

In order to determine the maximal transport capacity of the tubules for histamine (Tm), solutions of varying concentrations were infused at the same rate. Due to the strong physiological activity of histamine it is impossible to use large total amounts of histamine. This difficulty was avoided by infusing the stronger solutions during correspondingly shorter times. Thus the total amount infused was kept constant, and only the supply to the tubules per time unit was varied.

Results.

pH. In Fig. 1 are shown the results of two experiments with altogether 16 injections where $NaHCO_3$ was given to make the urine more alkaline during the course of the experiments. On the average the tubular excretion fraction with a urinary pH above 6.0 is as high as in the remaining cases. The two periods with a

Table I.

The apparent tubular excretion fraction of histamine at different histamine infusion rates.

Injection no.	Duration of injection sec.	Rate of injection		ATEF · 100	
		Histamine μg/min.	Phenol red μg/min.	Histamine	Phenol red
1	72	167	417	59	39.2
2	180	67	167	62	52.0
3	18	667	1,667	46	52.9
4	54	222	556	52	46.7
5	180	67	167	56	51.0
6	72	167	417	66	47.7
7	20	667	1,667	56	52.0
8	55	222	556	52	45.2

Every 15 min. 200 μg histamine and μg 500 phenol red are injected into the leg vein.

urinary pH around 8.0 followed upon an injection of 10 mg Diamox.

Tm. Table I contains values for the excretion of histamine found at different infusion rates in an experiment consisting of 8 injection periods. Apparently the dose per time unit has no influence on the uptake of histamine by the tubules within the limits for this experiment. Consequently it has not been possible to show any maximal tubular transport capacity by using an infusion rate of 667 μg per minute.

Brom cresol green. The tubules excrete brom cresol green by the same mechanism as phenol red (SPERBER 1954). The main data from two experiments with this compound are given in Table II. The secretion of histamine is apparently not influenced by the co-administration of brom cresol green while the concomitant secretion of phenol red is strongly depressed.

Priscoline. The results of two experiments with priscoline are shown in Table III. The secretion of histamine is consistently reduced after the injection of priscoline. This effect cannot be due to a change in renal blood-flow distribution as the concomitant secretion of phenol red is not diminished.

Cyanine. The influence of cyanine on the tubular excretion of histamine and phenol red has been investigated in four experiments with a total of seventeen injection periods. The effect of this compound was found to be rather weak. Only when the in-

Table II.

The influence of brom cresol green on the tubular excretion of histamine and phenol red.

Hen no.	Injection dose, μg			ATEF · 100	
	Histamine	Phenol red	Brom cresol green	Histamine	Phenol red
1	132	330	—	37	56
	132	330	132	41	—4
	132	330	132	56	—2
	132	330	—	35	47
2	—	510	—	—	44
	100	—	—	55	—
	204	510	—	56	52
	204	510	—	65	58
	192	480	960	59	9
	204	510	2,040	56	9
	204	510	—	49	51

jected dose of cyanine amounts to 1 mg the excretion of histamine is prevented. High doses of cyanine depress also the excretion of phenol red, making this substance less suitable for the above experiments.

Discussion.

At least two functionally discrete renal tubular secretory mechanisms have been described. Thus all organic acids known to be actively secreted by the renal tubules are usually considered to be transported by a common mechanism. They generally depress the excretion of one another, competing for secretion by the renal tubular cells.

The second mechanism (SPERBER 1947, 1948 b; RENNICK et al. 1947; BEYER et al. 1950) is concerned with the transport of organic bases.[1]

The basic cyanine dye # 863 has been described as a potent inhibitor of the tubular transport mechanism for organic bases by PETERS et al. (1955), although unequivocal evidence of renal tubular excretion of cyanine itself has not been obtained (RENNICK, KANDEL and PETERS 1956). Thus the tubular transport of N'-

[1] For a survey of the compounds known to be secreted by the tubules in the chicken see SPERBER 1954.

Table III.

*The influence of priscoline on the tubular excretion of histamine and phenol
red.*

Hen no.	Injection dose, μg			ATEF · 100	
	Histamine	Phenol red	Priscoline	Histamine	Phenol red
1	132	330	—	31	37
	132	330	687	7.5	47
	132	330	687	12.5	79
	132	330	—	46	74
	132	330	687	11	64
	132	330	687	11	84
	132	330	—	37	56
2	132	330	—	46	49
	132	330	687	11	44
	132	330	687	5	52
	132	330	—	53	53

methylnicotineamide (NMN) and Tetraethylammonium (TEA)
is inhibited both in dog and chicken (for a list of references see
RENNICK et al. 1956). RENNICK (1956) has also found an inhibi-
tion by cyanine of the excretion of choline in the chicken.

The excretion of priscoline which is excreted by the tubules in
the dog (ORLOFF, ARONOW and BERLINER 1953) is also inhibited
at least partly by cyanine (PETERS and KANDEL 1955) but the ab-
sence of mutual interference between the transport of priscoline
and NMN led to suggestions about a third transport mechanism
(ORLOFF et al. 1953). LE SHER and SHIDEMAN (1954, 1956) also
advanced such an idea since they had been unable to demonstrate
that darstine shares the same transport mechanism as utilized by
NMN, p-amino-hippuric acid or carinamid. However, SPERBER
(unpublished results) has found that the tubular excretion of
NMN is inhibited by priscoline and darstine in the hen, and
PETERS and KANDEL (1955) have reported that darstine at high
plasma levels can inhibit the renal clearance of NMN in the dog.
The failure to demonstrate competition between similar compounds
for excretion may be due to differences in affinities for the carrier
mechanism which is also taken into consideration by ORLOFF
et al., and LE SHER and SHIDEMAN. It seems very likely that all
of the organic bases known to be excreted by renal tubular activity
are transported by the same mechanism.

The experiments presented in this report give evidence that the tubular excretion of histamine takes place through the transport mechanism which is utilized by other organic bases, excreted by the avian and mammalian tubules.

The absence of competition between histamine and brom cresol green shows that the transport mechanism for histamine is functionally distinct from that dealing with organic acids. The experiments with priscoline on the other hand indicate that histamine and priscoline compete for the same transport mechanism.

The depression of the histamine excretion by cyanine is weaker than that found in dogs by other authors for the tubular transport of TEA and NMN. However, RENNICK et al. (1956) found in the chicken a cyanine inhibition of TEA excretion which was more delayed and less striking than in the dog.

The highest doses of histamine used in the experiments presented here were not sufficient to determine a Tm value, but gave a secretory capacity of about 6 μmoles per minute. It must, however, be emphasised that this may reflect a high rate of uptake rather than of secretion, as the method used does not necessarily differentiate between these two processes. The rate of secretion seems, however, to be fairly high.

Summary.

1. The pH of the urine appears to have no influence upon the tubular excretion of histamine.

2. Tm for histamine is shown to be comparatively high.

3. The excretion of histamine is not influenced by brom cresol green.

4. The excretion of histamine is depressed by priscoline and, at east partly, by cyanine.

References.

BEYER, K. H., H. F. RUSSO, S. R. GASS, K. M. WILHOYTE, and A. A. PITT, Amer. J. Physiol. 1950. *160*. 311.

KEMPTON, R. T., J. Cell Comp. Physiol. 1939. *14*. 73.

LE SHER, D. A., and F. E. SHIDEMAN, J. Pharmacol. 1954. *110*. 32.

LE SHER, D. A., and F. E. SHIDEMAN, J. Pharmacol. 1956. *118*. 407.

LINDAHL, K., and I. SPERBER, Acta physiol. scand. 1956. *36*. 13.

ORLOFF J., L. ARONOW, and R. W. BERLINER, J. Pharmacol. 1953. *109*. 214.

PETERS, L., K. J. FENTON, M. L. WOLF, and A. KANDEL, J. Pharmacol. 1955. *113*. 148.

PETERS, L., and A. KANDEL, Fed. Proc. 1955. *14*. 377.

RENNICK, B. R., Fed. Proc. 1956. *15*. 472.

RENNICK, B. R., A. KANDEL, and L. PETERS, J. Pharmacol. 1956. *118*. 204.

RENNICK, B. R., K. G. MOE, S. W. HOOBLER, R. NELIGH, and R. H. LYONS, Fed. Proc. 1947. *6*. 364, and J. Pharmacol. 1947. *91*. 210.

SMITH, H. N., The Kidney, Oxford University Press. 1951. Chapter VI.

SPERBER, I., Int. Physiol. Congr. Abstr. XVII. Oxford. 1947.

SPERBER, I., Ann. Roy. Agric. Coll. Sweden. 1948. *15*. 317.

SPERBER, I., Ann. Roy. Agric. Coll. Sweden. 1948 b. *16*. 49.

SPERBER, I., Arch int. pharmacodyn. 1954. *97*. 221.

The Influence of Tobacco Smoking, and Increased Initial Carbon Monoxide Concentration on Results of Sjöstrand's Method of Total Hemoglobin Determination.

By

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SJÖSTRAND'S modification of the carbon monoxide method for blood volume determination (SJÖSTRAND 1948) is based upon the assumption that the increase in COHb concentration upon the administration of CO to a rebreathing system can be calculated from the change in the composition of the gas in the rebreathing system. This calculation is made according to Haldane's Law I.

A non-smoker has an initial COHb concentration of between 0.3 —1.0 per cent. In smokers, the initial COHb concentration may amount to several per cent. When Sjöstrand's method is used, 15—20 ml of CO is taken up by the hemoglobin which results in an increase in the COHb concentration of 1—4 per cent.

It has been stated that in smokers with a high initial COHb concentration values of total hemoglobin carry larger experimental errors than those encountered in non-smokers, since the error of the CO meter is directly proportional to the measured CO concentration (SJÖSTRAND 1948). However, we found quite regularly abnormally high values of total hemoglobin in smokers. These results could not be ascribed to technical errors.

In this paper experiments are reported which were planned to help answering the following question: Are the high initial COHb values of smokers responsible for the abnormal results of total hemoglobin determinations, or is there some other effect of smoking?

Methods and Material.

Details concerning the determination of total hemoglobin are contained in a thesis by Wiklander (1956). In the calculations he used a value of the factor M in Haldane's Law I of 231. In the present paper the value 225 was used. The difference is unimportant.

The investigation was made on 13 healthy subjects who were moderate to heavy cigarette smokers, and 14 healthy non-smokers.

Three groups of experiments were performed, A, B, and C. One subject is included both in groups A and B.

A. On two or three consecutive days determinations of total hemoglobin were made on 6 of the smokers. Five of them had stopped smoking a short time before the first test, the sixth was allowed to smoke until the beginning of the first experiment. All abstained from smoking throughout the experiments.

B. On six smokers four determinations were made on consecutive days. 1. An ordinary determination. 2. A determination preceded by two 15 minutes periods of O_2 breathing in a closed system. 3. A determination preceded by 20 minutes of O_2 breathing in an open system. 4. An ordinary determination after one day's abstinence from smoking.

C. Two determinations of total hemoglobin, separated by some 10 minutes, were made on 14 non-smokers. On five of these an analysis was made of the O_2 and the CO content, and the O_2 capacity in venous blood samples determined at the end of the first and last rebreathing period in every experiment.

Gas and blood analyses were carried out as described in earlier papers by DAHLSTRÖM (1955, 1956). Ferricyanide was used as a reagent. Besides the CO determination, the O_2 capacity was determined on every blood sample with the Van Slyke apparatus. Then the blood sample was saturated in a flask with air for 20 minutes. The O_2 capacity determination was carried out in triplicate. The hemoglobin of an aliquot blood sample was determined spectrophotometrically as alkaline hematin. A Coleman spectrophotometer was used.

Table I.

Results of Experiments, Group A.

Determinations on smokers: 1, having smoked 1—3 hours prior to the determination, 2 and 3 after 1 and 2 days abstinence from smoking respectively.

No.	First bag		Third bag		Increase of COHb	Absorbed amount of CO	Total Hemo-globin
	COHb	O_2	COHb	O_2			
	%	%	%	%	%	ml	g
1	1.23	98.6	2.90	95.9	1.67	16.56	703
2	0.50	97.2	2.46	97.0	1.96	16.56	599
3	0.45	98.2	2.52	96.7	2.07	16.51	565
1	2.40	95.3	3.76	94.8	1.36	16.23	846
2	0.53	94.8	2.30	96.6	1.77	16.68	668
1	1.39	96.8	2.58	93.1	1.19	16.63	991
2	0.58	95.7	1.91	95.6	1.33	16.83	897
1	2.54	95.6	4.20	95.7	1.66	25.27	1,079
2	0.82	97.1	2.07	96.9	1.25	17.03	966
1	2.15	96.2	3.24	90.4	1.09	16.87	1,097
2	0.76	95.9	2.01	90.1	1.25	17.15	973
1	3.03	97.4	4.48	96.6	1.45	15.82	774
2	0.45	97.3	2.52	93.8	2.07	16.46	564
3	0.37	97.2	2.48	95.8	2.11	16.43	552

Results.

In group A (Table I), *i. e.* smokers in which determinations were made on subsequent days, the initial values of the COHb and total hemoglobin show a decrease on subsequent days. The difference in total hemoglobin between the first and the last determination totals from 9 to 21 per cent in the subjects who stopped smoking two hours before the test, and 29 per cent in the subject who smoked immediately before the test was begun (last subject in Table I).

In group B (Table II), the first and fourth experiment showed the same tendency as the experiments in group A: In 4 cases out of 6 the value of total hemoglobin obtained after one day's abstinence from smoking were between 6 and 27 per cent lower, while in 2 cases no significant difference was observed.

The results of the experiments (2) in group B show the effect of thirty minutes of O_2 breathing in a closed system preliminary to the regular determinations. The values were then reduced by 0 to 30 per cent, as compared with the results after smoking. Compared with the values obtained after abstinence from smoking (4) they

Table II.

Results of Experiments, Group B.

1, 2 and, 3 denote determinations on smoking: 1, regular determination, 2, after 30 minutes extra O_2 breathing, closed system, 3, after 20 minutes extra O_2 breathing, open system, 4, determination after 17—20 hours abstinence from smoking.

No.	First bag		Third bag		Increase of COHb	Absorbed amount of CO	Total Hemo-globin
	COHb	O_2	COHb	O_2			
	%	%	%	%	%	ml	g
1	0.98	96.3	3.34	95.9	2.36	25.52	767
2	0.83	96.2	3.28	94.5	2.45	25.30	650
3	1.06	98.4	3.34	96.8	2.28	25.48	792
4	0.76	96.8	3.08	93.7	2.32	25.14	768
1	1.83	95.5	3.84	96.4	2.01	25.07	884
2	1.41	93.8	3.09	95.9	1.68	16.44	693
3	1.42	96.8	3.89	93.4	2.47	24.88	714
4	0.69	96.2	3.29	92.5	2.60	25.51	696
1	1.96	97.4	5.81	96.7	3.85	23.47	432
2	1.36	94.2	5.23	96.4	3.87	23.71	434
3	1.71	98.1	5.87	93.3	4.16	23.42	399
4	0.74	97.1	5.21	96.7	4.47	23.84	378
1	2.31	95.9	3.86	96.4	1.55	16.19	741
2	1.72	94.6	3.57	92.0	1.85	16.59	636
3	2.17	97.0	3.81	96.4	1.64	16.26	703
4	1.04	95.0	2.76	92.0	1.72	16.87	695
1	3.12	95.9	5.42	94.3	2.30	24.44	753
2	2.94	95.5	5.64	92.3	2.70	24.00	630
3	2.18	97.7	4.54	97.2	2.36	24.80	744
4	1.07	96.0	3.36	95.4	2.29	24.81	768
1	3.03	97.4	4.48	96.6	1.45	15.82	774
2	0.61	94.6	2.70	91.2	2.09	15.71	540
3	1.48	97.0	3.51	94.2	2.03	16.25	568
4	0.45	97.3	2.52	93.8	2.07	16.46	564

were higher in 3 cases, lower in 4, and the same in one. The difference ranged from — 18 to + 15 per cent. When the regular determinations of total hemoglobin were preceded by 20 minutes of O_2 breathing in an open system (3), the total hemoglobin level differed only slightly from the values obtained after abstinence from smoking; the differences were between — 5 and + 3 per cent.

In group C (Table III), with two determinations in immediate sequence, the second determination showed the total hemoglobin value to be smaller in 12 cases, and unchanged in 2 cases. The difference was tested by the t-test, and is almost significant (0.02 > P > 0.01) (range 0 to 6 per cent, mean 3 per cent).

The results of the determinations in group C when blood analyses

Table III.

Results of Experiments, Group C.

1, 2, denote 1st and 2nd experiment.

No.	First bag		Third bag		Increase of COHb	Absorbed amount of CO	Total Hemo- globin
	COHb	O₂	COHb	O₂			
	%	%	%	%	%	ml	g
1	0.77	97.0	3.03	90.6	2.26	16.20	508
2	2.50	97.1	4.70	92.6	2.20	15.54	501
1	0.50	96.6	2.38	96.1	1.88	17.17	648
2	1.92	97.0	3.79	92.2	1.87	16.22	615
1	0.85	95.7	3.22	90.8	2.37	16.28	486
2	2.43	96.3	4.70	95.9	2.27	15.56	486
1	0.77	96.1	2.59	95.4	1.82	16.22	632
2	2.31	95.7	4.13	94.4	1.82	15.66	610
1	0.57	96.2	2.26	93.5	1.69	16.17	678
2	2.01	96.8	3.71	93.4	1.70	15.66	653
1	0.69	96.2	2.30	92.5	1.61	15.95	702
2	2.02	97.1	3.69	95.9	1.67	15.98	678
1	0.63	96.7	2.22	95.5	1.59	16.06	716
2	2.07	97.4	3.66	91.3	1.59	15.63	697
1	0.73	88.0	2.29	93.7	1.56	16.26	739
2	2.08	97.7	3.69	93.8	1.61	15.77	695
1	0.65	96.7	2.11	97.0	1.46	16.52	802
2	1.98	97.2	3.48	96.5	1.50	15.98	765
1	0.43	96.4	3.43	93.6	3.00	24.77	585
2	2.50	97.4	5.30	96.6	2.80	23.94	584
1	0.84	96.2	3.51	93.0	2.67	24.89	661
2	3.00	96.5	5.76	94.8	2.76	23.80	611
1	0.64	95.7	2.84	93.0	2.20	25.47	821
2	2.53	93.9	4.67	92.9	2.14	24.78	821
1	0.63	95.8	2.87	97.2	2.24	25.45	806
2	2.34	95.7	4.69	94.8	2.35	24.69	745
1	0.43	96.3	2.26	94.0	1.83	16.69	647
2	1.99	97.1	3.80	95.0	1.81	16.10	631

were carried out are given in Table IV. The O₂ capacity is on an average 0.43 volumes per cent lower in blood sample No. 2, which corresponds to an increase in COHb of about 2.5 per cent. According to CO analyses of the blood samples the COHb has on an average increased by 2.82 per cent. The fact that an exact agreement was not obtained may partly be due to some CO having left the blood sample when it was saturated with air. It will be seen from the photometer readings that very little evaporation took

Table IV.

Results of Experiments on last five Subjects of Group C.

|	Calculated values (M = 225)						Blood analyses								
	$COHb_1$	O_2	$COHb_3$	O_2	Absorbed amount	Tot Hb	$COHb_1$	Photometer reading	$COHb_3$	Photometer reading	O_2 cap	Photometer reading	O_2 cap	Photometer reading	L
	%	%	%	%	ml	g	%		%		vol. %		vol. %		
1	0.43	96.4	3.43	93.6	24.77	585	0.46	229	4.14	239	15.84	239	15.53	243	279
2	2.50	97.4	5.30	96.6	23.94	584	2.61	244	6.19	239	15.08	245	15.04	238	290
1	0.84	96.2	3.51	93.0	24.89	661	0.94	270	4.33	267	17.35	267	16.64	281	288
2	3.00	96.5	5.76	94.8	23.80	611	2.65	269	6.68	266	16.69	258	16.14	285	308
1	0.64	95.7	2.84	93.0	25.47	821	0.66	281	3.02	283	18.49	279	18.32	288	242
2	2.53	93.9	4.67	92.9	24.78	821	2.75	289	5.04	276	18.91	289	18.21	280	242
1	0.63	95.8	2.87	97.2	25.45	806	0.75	261	2.87	249	18.97	265	17.78	247	213
2	2.34	95.7	4.69	94.8	24.69	745	2.56	248	5.00	258	18.02	256	17.96	260	235
1	0.43	96.3	2.26	94.0	16.69	647	0.50	224	2.30	203	16.33	221	15.35	207	219
2	1.99	97.1	3.80	95.0	16.10	631	1.93	191	4.45	202	14.21	191	14.66	208	315
Mean							1.58	250.6	4.40	248.2	16.99	251.0	16.56	253.7	263
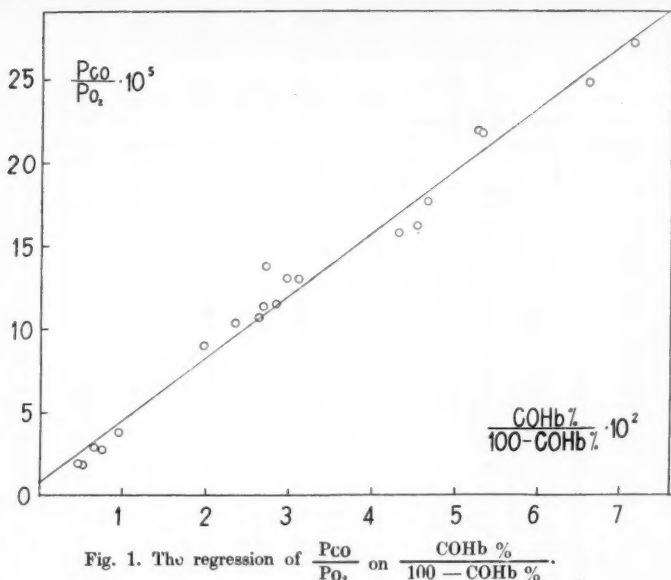
Fig. 1. The regression of $\dfrac{P_{CO}}{P_{O_2}}$ on $\dfrac{COHb\ \%}{100 - COHb\ \%}$.

place during the analytic procedure; these readings however, show a rather extensive distribution.

The results may be shown diagrammatically according to Fig. 1, where $\dfrac{P_{CO}}{P_{O_2}}$ and $\dfrac{COHb\ \%}{100 - COHb\ \%}$ have been chosen as coordinates. Thus the one coordinate has been calculated from the results of analyses of rebreathed gas, the other from analyses of blood samples. As is seen in the Figure the obtained points are only slightly scattered around a line.

The regression of $\dfrac{P_{CO}}{P_{O_2}}$ on $\dfrac{COHb\ \%}{100 - COHb\ \%}$ is

$$\frac{P_{CO}}{P_{O_2}} = \frac{1 \cdot COHb\ \%}{266 \cdot (100 - COHb\ \%)} + 0.807 \cdot 10^{-5}.$$

The correlation coefficient is 0.99. From the equation it will be seen that the value of the factor M is 266. The regression line does not deviate significantly from the origin (P < 0.05). The values of M however vary between 198 and 280.

Discussion.

The total hemoglobin of the subjects of this investigation presumably remained unchanged throughout the experiments. The changes observed in the total hemoglobin must, therefore, be due to an erroneous interpretation of the data obtained.

In group A less CO was absorbed when the initial COHb was high in 4 of 5 comparable cases; in group B (on comparison of experiments 1 and 4) in 4 of 5 cases, and in group C in 13 of 14 cases. This was to be expected as the same amount of CO was administered to the rebreathing system in both cases, but more was taken away with bags Nos. 2 and 3 in an experiment when the COHb was initially high. Thus in such a case the increase of COHb should be less, which is also the case in group A and B, *but not* in group C.

In this group of non-smokers the increase is greater in 6 cases, less in 6 cases and unchanged in 2 cases. The average increase in the first determination is 2.01 per cent and in the second determination 2.01 per cent. Thus the calculated values of COHb revealed the same increase in both experiments, although a smaller quantity of CO was absorbed in the second experiment. We are therefore obliged to assume that the COHb values were erroneously estimated, so that the difference between the initial and final COHb concentrations has been overestimated in the experiments with high initial COHb, or underestimated in the experiments with a low initial COHb.

The factor L, which is an expression of the relative affinities of CO and O_2 for hemoglobin during the formation of an additional amount of COHb (DAHLSTRÖM 1955), has been calculated and is given in Table IV. The value of this factor is greater in the second determination for each person in 4 of the 5 cases, and in one case is the same in both determinations. The difference is not significant, but nevertheless indicates that the error in the estimation of the total Hb is to be found in the estimation of the COHb rather than in the absorbed amount of CO. In that case, where the factor L was similar in both experiments, the total Hb was also the same in both experiments which further supports this assumption.

The influence on the CO-COHb equilibrium of the "distribution factor" might lead to an erroneous estimation of COHb from an analysis of rebreathed gas (DAHLSTRÖM 1956). It might be questioned

as to whether the discrepancies in the total Hb in group C (Table III) could be explained with the help of this hypothesis. If a low value of the constant M is used when calculating the lowest COHb values (the first determination in the first experiment) this would denote a correction in such a way that the total Hb would be the same in both the first and the second experiment. However, our determinations of the factor M have not been accurate enough for the result to be used as a recount of the results in group C.

The fact that greater values of L were obtained in experiment No. 2 (Table IV) on the four experimental subjects who had the lower total Hb in the second experiment might signify the Pco in these determinations being underestimated *i. e.* being greater in the blood than was calculated from the composition of the re-breathed gas. Further determinations are necessary to determine how great this difference is and at which COHb values the difference is greatest.

It should be mentioned that the use of another value of the factor M throughout does not really change the final results in this respect. If instead one calculates the total Hb with a very low value of M the differences between the increase of COHb in the first and second experiments is insignificant. (For M = 135 a difference of only 1 per cent is obtained which is insufficient to remove the discrepancies).

As will be seen in Table I and II the absorbed quantities of CO were less in those cases where the initial COHb was highest among smokers (groups A and B). Even the increase in COHb was less in the latter, but so much less that the calculated values of the total Hb were higher in those experiments where the initial COHb was high.

In experiments on smokers in which extra 15 minute periods of O_2 breathing in a closed system preceded the total hemoglobin determination, lower values were obtained than in determinations made without these periods. This indicates that much of the effect of smoking can be eliminated by 30 minutes of O_2 breathing.

If a smoker breathed O_2 from an open system for 20 minutes before the determination of total hemoglobin, the results were almost identical with those obtained after abstinence from smoking. This implies that practically the whole effect of smoking can be eliminated in this way, although the initial COHb value remains high.

A possible explanation of this finding is that the distribution of

the ventilation/perfusion ratios of the alveoli is rendered more uneven by smoking (nicotine action on pulmonary blood vessels?). The COHb values would then be underestimated to a greater extent than is usual with the present method, and likewise the difference between the initial and final COHb values. The total hemoglobin would then be overestimated.

Tentatively, it may be concluded that the CO-COHb equilibrium is influenced by the "distribution factor". The influence of posture (DAHLSTROM 1956), of increased initial COHb, and the effect of tobacco smoking could possibly be interpreted according to this hypothesis.

In this investigation smoking was found to influence the values of total hemoglobin by up to 30 per cent. In the data of DAHLSTRÖM (1955) there were smokers who, notwithstanding the instructions given them, had probably smoked before the determinations. The values of L in two of those subjects deviated from a "normal" value of 225 by 80 and 57 per cent respectively, while in the remaining cases the difference was less than 30 per cent. The underlying cause of the two largest deviations remains obscure, but the variations in the rest of the series are in conformity with the present findings in smokers. Another possible explanation of these and other deviations is found in recently published data which have been interpreted as evidence of oxidation of CO in the erythrocytes (ALLEN and ROOT 1957). It is natural to suppose that this oxidation is temporarily accelerated when O_2 breathing begins, only to again assume a slower rate.

A practical conclusion of our findings is that SJÖSTRAND's method of total hemoglobin determination can be applied to smokers, provided they are made to breathe O_2 from an open system for a preliminary 20-minute period. Moreover, duplicate determinations in immediate sequence are practically unaffected by the increase in the initial COHb concentration.

Theoretically, results of total hemoglobin determinations might be influenced by prevailing pulmonary circulatory conditions. This might have been the cause of additional variations in results with the CO method (WIKLANDER 1956), as compared with the results of determinations with Evans Blue or P^{32}.

Summary.

1. Determinations of total hemoglobin according to SJÖSTRAND's modification of the CO method were carried out in three groups of experiments: A. On smokers on consecutive days, when the subjects had stopped smoking before the first test. B. On smokers, with additional O_2 breathing immediately before the test. C. On non-smokers with two determinations separated by some 10 minutes. In a part of this group venous blood samples were drawn during the procedure and analyzed for CO.

2. It was observed that the effect of smoking upon results of total hemoglobin determinations can be eliminated by a 20 minute period of O_2 breathing from an open system, and that increased initial COHb, as obtained in repeated determinations, does not substantially influence the results.

3. The results are discussed and interpreted with regard to the influence of the distribution of the ventilation/perfusion ratios of the alveoli.

References.

ALLEN, T. H. and W. S. ROOT, J. appl. Physiol. 1957. *10*. 186.
CARLSTEN, A., A. HOLMGREN, K. LINROTH, T. SJÖSTRAND and G. STRÖM. Acta physiol. scand. 1954. *31*. 62.
DAHLSTRÖM, H., Acta physiol. scand. 1955. *33*. 296.
DAHLSTRÖM, H., Acta physiol. scand. 1956. *36*. 256.
SJÖSTRAND, T., Acta physiol. scand. 1948. *16*. 201, 211.
WIKLANDER, O., Acta chir. scand. 1956. Suppl. 208.

Effect of Heart Rate on Cardiac Work, Myocardial Oxygen Consumption and Coronary Blood Flow in the Dog.

By

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The changes in cardiac frequency which occur in the intact man or animal are usually accompanied by large changes in cardiac output and vascular pressures. It is difficult to evaluate to what extent the hemodynamic changes are caused by the altered heart rate *per se* and to what extent they are caused by other factors. In the present study the heart rate was varied by direct electrical stimulation of the heart. The relation between ventricular filling pressure and ventricular work was observed at various rates. Myocardial oxygen consumption and coronary blood flow were also measured. The role of myocardial oxygen consumption *versus* mechanical factors in determining coronary blood flow was evaluated. A preliminary report was given in 1955 (DUFF, BERGLUND, and BORST).

Method.

The dogs were premedicated with morphine (1.6—2.7 mg/kg) and anesthetized with chloralose (0.05—0.1 g/kg) and urethane (0.5—1.0 g/kg). After thoracotomy ventilation was maintained by a constant-

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volume pump. Flowmeters were introduced for measurement of systemic blood flow and coronary blood flow.

Control of Heart Rate.

Three methods were used: 1) *Sinus node crushing and right atrial stimulation* (3 dogs). The area of the sino-auricular node was crushed. Sometimes the heart rate and electrocardiogram returned to the control state within a few minutes; a larger area was then crushed. The rate was then raised to desired levels by electrical stimulation of the right atrium. In order to maintain constant rates during blood infusion, bilateral vagotomy was necessary. The lowest heart rates obtained after sinus node crushing and vagotomy were 105—110 per minute. — 2) *Vagus nerve stimulation and electrical stimulation of the heart* (3 dogs). The distal end of the cut right and left vagus nerve was electrically stimulated with a Grass stimulator. While constant vagal stimulation was maintained, the heart rate was raised to desired levels by electrical stimulation of one atrium or ventricle. At intervals the atrial stimulation was interrupted to check that the vagal stimulation still was effective. — 3) *Surgical production of atrio-ventricular block and electrical stimulation of the ventricles* (25 dogs). Atrio-ventricular block was produced by the method described by STARZL and GAERTNER (1955). During occlusion of both venae cavae the right atrium was opened and an incision was made into the area of the His bundle. As this incision sometimes extended into other cavities, the method was modified (by H. G. B.): a heavy silk ligature was tied around the bundle area. This procedure was followed by a short period of ventricular standstill and a slow idioventricular rate as seen on electrocardiograms or by direct observation of the heart. The ventricular rate was raised to desired levels by electrical stimulation via an electrode implanted in the right ventricular wall near the anterior part of the septum. Regular rates from 40 up to at least 200 per minute could be obtained. Coronary blood flow was measured only in these dogs. All the illustrations are from dogs in which this latter procedure was used.

Measurements.

The method has been described and discussed in detail (SARNOFF and BERGLUND, 1954, CASE et. al. 1954, BERGLUND, 1955). Pressures were obtained via catheters in the right atrium, pulmonary artery, left atrium, and thoracic aorta. Systemic blood flow (cardiac output minus coronary blood flow) was measured with an electronic flowmeter[1] (SARNOFF and BERGLUND, 1953) and (in 13 dogs) left coronary artery blood flow was measured with an electrically recording rotameter (SHIPLEY and WILSON, 1951). All values were recorded on a direct-writing oscillograph. Heart rates were read from a tachometer[2] connected to the pulmonary artery manometer. Coronary sinus blood samples were obtained through a short Goodale-Lubin catheter, introduced through a jugular vein or through the right auricle (14 dogs).

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[1] Electroturbinometer, Potter Aeronautical Co., Newark, N. J.
[2] Waters Conley Co., Rochester, Minn.

Blood oxygen contents and capacities were analyzed by the method of Van Slyke and Neill.

The stroke work of each ventricle was obtained by multiplying the stroke volume by the difference between mean arterial pressure and mean atrial pressure. Mean left atrial and end-diastolic left ventricular pressures were found to correlate closely at both low and high cardiac frequencies. "Myocardial oxygen consumption" was calculated as the product of the arterio-coronary sinus oxygen difference and the coronary blood flow (11 dogs).

Two types of study were done: 1) *Heart rate changes with constant blood volume* (See Fig. 1). The rate was increased in steps and held at each level for a constant period, usually one minute. After having reached levels of up to 280 per minute, the rate was usually returned to low values in steps with similar time intervals. Pressures and flows had reached a steady state at the end of these intervals, and blood samples were obtained at that time. 2) *"Ventricular function curves" at various heart rate levels* (See Fig. 2). The blood volume of the dog and thus the ventricular filling pressures were increased in steps by infusion of donor dog blood and dextran from a reservoir connected with a femoral vein. This procedure was repeated at various heart rates. Ventricular function curves for each ventricle were obtained by plotting the stroke work of the ventricle against the filling pressure (SARNOFF and BERGLUND, 1954).

Results.

I. *Effect of variations in heart rate at a constant blood volume.*

Figure 1 shows the typical response. With increasing rate there was a moderate increase in cardiac output, followed at higher rates by a levelling and a decrease. The stroke volume fell progressively as the frequency was increased. Aortic mean pressure rose slightly while the pulse pressure decreased. Myocardial oxygen consumption increased markedly. Coronary blood flow increased roughly in proportion to the myocardial oxygen consumption. This increase occurred also at high rates where mean arterial pressure was unchanged. Consequently, the coronary vascural resistance decreased with rate, even at high rates.

This type of experiment was done 37 times in 17 dogs. The maximum cardiac outputs in the different dogs were obtained at rates between 90 and 180 per minute, mostly 100—150 per minute. The increase in output (above that at a rate of 60 per minute) was 0—73 per cent; in 12 of the 17 dogs the increase was more than 30 per cent. In 8 dogs this procedure was done at low, medium, and high blood volumes; the above pattern was the same at different blood volumes. Similar blood pressure patterns were also obtained
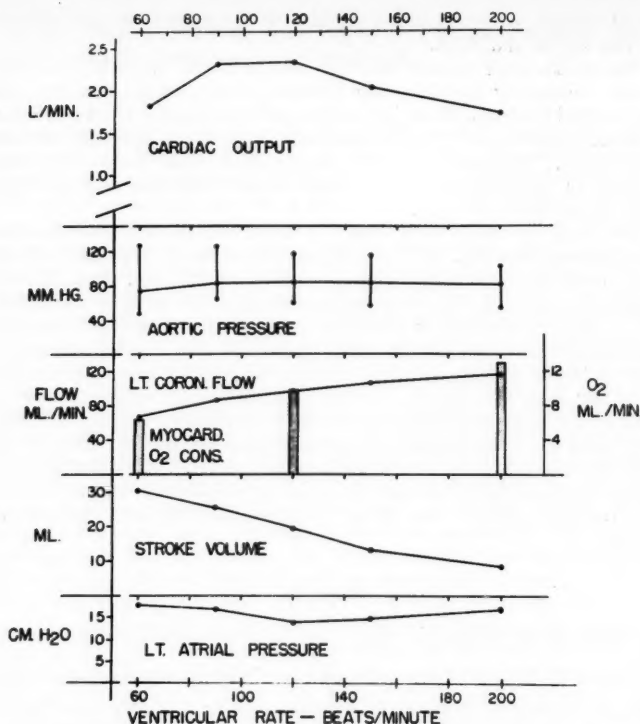
Fig. 1. Effect of heart rate on cardiac output, aortic pressure, myocardial oxygen consumption, coronary flow, stroke volume and left atrial pressure. Dog 214, weight 22 kg.

in 2 dogs in which cardiac frequency was varied only by graded vagal stimulation and no flowmeters were introduced.

II. *Ventricular function.*

Figure 2 shows a typical set of left ventricular function curves obtained at various heart rates. The higher the rate, the lower is the stroke work. This type of result was obtained in 16 dogs. At heart rates below 100 the differences between curves were not consistent in all experiments. Similarly, the stroke volume at any filling pressure was higher at low frequencies than at high frequencies. The maximum cardiac outputs in this type of experiment were
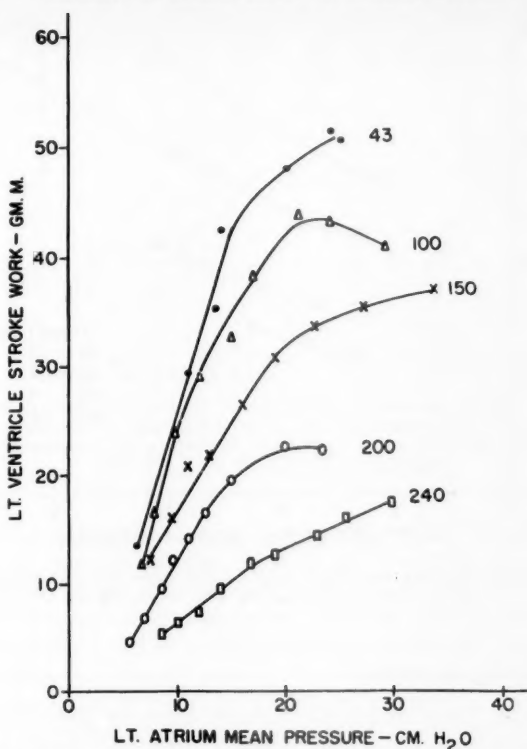
Fig. 2. Ventricular function curves at various heart rates. Dog 168, weight 17 kg.

obtained at rates between 120 and 190 per minute. The right ventricular function curves showed the same type of response.

III. *Myocardial oxygen consumption.*

Figure 3 shows the relation between myocardial oxygen consumption and ventricular work at various heart rates. At any given work level the oxygen consumption increases with heart rate. The relation between arterial pressure and cardiac output was approximately the same in the different runs. Thus, work efficiency of the heart is lower at high heart rates.
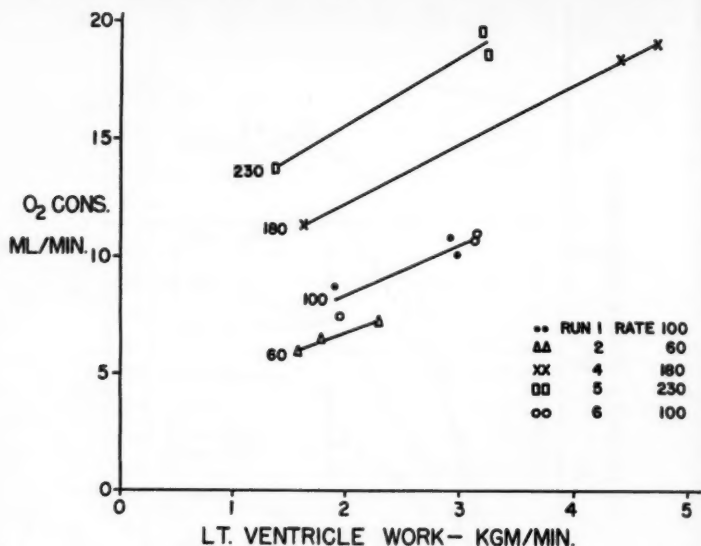
Fig. 3. Relation between myocardial oxygen consumption and left ventricle work at various heart rates. Dog 228, weight 19 kg.

IV. Coronary blood flow.

Figure 4 shows the relation between coronary blood flow and "effective coronary perfusion pressure" (left) and between coronary flow and myocardial oxygen consumption (right) at various heart rates. It is seen that at a given perfusion pressure the coronary flow is larger with high frequencies than with low frequencies, in spite of the decreased time per minute available for coronary perfusion. The average coronary vascular resistance at a rate of 230 is less than half of that at 60 per minute. On the other hand, the relation between coronary flow and myocardial oxygen consumption (right) is the same at all the heart rates studied.

V. Coronary sinus oxygen saturation.

It was attempted to maintain arterial oxygen saturation and hematocrit constant throughout the experiment. In those experiments in which coronary blood flow and coronary sinus saturation were studied together, the sinus saturation was somewhat lower
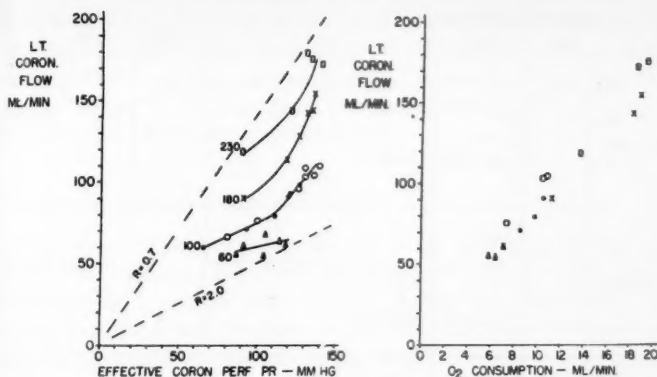
Fig. 4. Relationship of coronary flow to effective perfusion pressure (pressure difference between coronary artery ostium and right atrium) and to myocardial oxygen consumption. Same experiment as in Fig. 3. Interrupted lines denote resistance of 0.7 and 2.0 mm Hg/ml flow/min.

at high rates (25—35 per cent) than at low rates (30—40 per cent), but never below 25 per cent. In three other experiments without cannulation of the coronary artery this trend could not be confirmed; it is possible that the artery cannulation increased the tendency towards a fall of coronary sinus saturation. In these three experiments the coronary sinus content of lactate and of pyruvate was determined[1]; no consistent change with heart rate was found. These data indicate that anaerobic metabolism was not increased at high heart rates.

VI. *Effect of increased heart rate in the presence of coronary constriction.*

In two experiments the coronary in-flow tubing was constricted by a screw clamp, so as to simulate mild coronary stenosis, and the heart rate was then raised. Elevations of the rate up to 130 per minute were accompanied by changes similar to those shown in Fig. 1, namely a rise in cardiac output, aortic pressure, and coronary blood flow. However, when the rate was elevated further, to 150—160 per minute (see Fig. 5), there was no further rise in coronary flow. At these frequencies signs of left ventricular failure developed (Case et al. 1954), namely a fall in cardiac output and

[1] The authors are grateful to Mrs. P. Osborn and Miss A. Nylander at the Massachusetts General Hospital for these determinations.
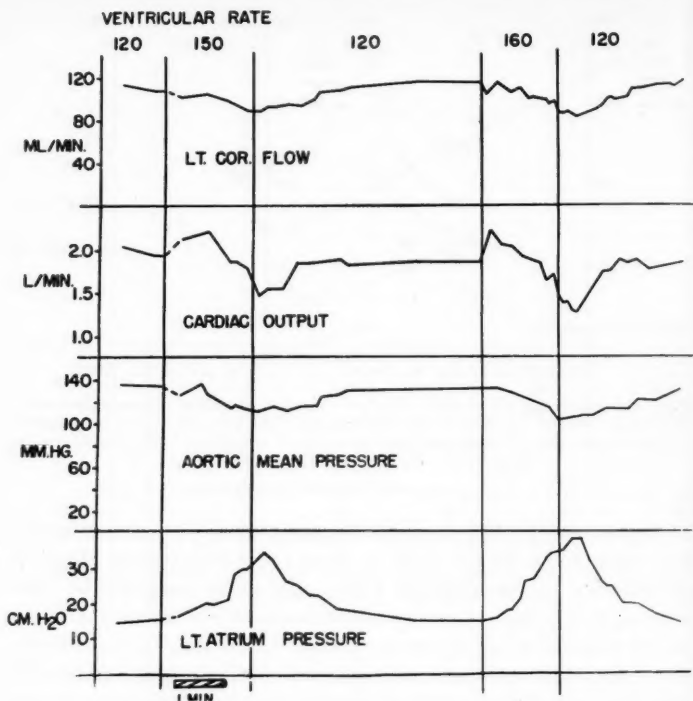
Fig. 5. Effect of changes in heart rate *in the presence of coronary stenosis*. Same dog as in Figs. 3 and 4 but with the coronary inflow tubing narrowed by a screw clamp. The ventricular rates are at the top of the figure. See text.

aortic pressure together with a rise in left atrial pressure and a gradual fall of coronary flow. By simply returning the heart rate to a lower level, the signs of coronary insufficiency were reversed. Apparently there was in this situation a discrepancy between the oxygen demand and the coronary blood flow at high heart rates, leading to myocardial hypoxia.

Discussion.

In this study the heart rate was controlled by means which do not employ alterations of hormone activity, temperature or nerve stimulation. The obtained responses of the ventricles should there-

fore be a direct function of the heart rate. This is supported by the fact that the results were similar when three different methods were used for varying the heart rate. The results bring up interesting points on regulation of cardiac work, myocardial oxygen consumption, and coronary blood flow.

Changes in cardiac output and work.

The changes in stroke volume, cardiac output, and cardiac work with increased heart rate are markedly different from those occurring with similar rate changes during exercise (ÅSTRAND, 1952, ASMUSSEN and NIELSEN, 1955, BARGER et al., 1956); there was only a moderate rise in cardiac output and a progressive fall in stroke volume. During exercise, factors other than increased frequency contribute to the large increases in cardiac output. These factors probably include changes in vasomotor tone and increased myocardial contractility due to increased sympatho-adrenal activity. Further support for this is given by the findings that in dogs cardiac work and output increased more during exercise than during similar cardiac rates induced by electrical stimulation of the heart (RUSHMER, 1956), while in man atropine-induced cardio-acceleration does not raise cardiac output (GORLIN, 1957).

Ventricular function curves, i. e. ventricular stroke work per unit of filling pressure, were markedly lowered with increased rate. This means either that stroke work at a given diastolic volume is decreased with higher rates, or that the diastolic volume at a given filling pressure is less at higher rates, or both. Since most of the ventricular filling supposedly takes place in the early part of diastole (rapid filling phase), one would not expect any marked rate effect on diastolic volume with frequencies below 180 per minute. On the other hand, studies by ULLRICH et al., (1954) and BUCKLEY et. al. (1955) demonstrated that diastolic filling characteristics vary with frequency so that diastolic size at a given filling pressure is smaller at high frequencies. This could explain, at least in part, the lowering of ventricular function curves with increased frequency. Other experiments, in which the "isometric tension development" of an in situ segment of the ventricular wall was studied at a fixed fibre length, demonstrated no change in "contractility" with cardiac frequencies between 65—90 and 260 per minute (COTTEN, 1953). This indicates that heart rate

per se does not alter the relation between diastolic volume and ventricular stroke work. It is therefore concluded that the lowering of the function curves with increased rate is largely due to altered filling conditions, namely an increased impedance to filling.

Myocardial oxygen consumption.

The mechanical factors in the heart which determine myocardial oxygen consumption were studied qualitatively by ROHDE (1912), EVANS and MATSUOKA (1914) and GOLLWITZER-MEIER and KRÜGER (1936). Quantitative studies on the relation between contraction time, ventricular pressure or tension and oxygen consumption have recently been reported by SARNOFF et al. (1958). They found that the oxygen consumption was linearly related to the "total tension index", *i. e.* the product of systolic time per minute and systolic ventricular pressure or tension. At a given level of work per minute, increased heart rate is accompanied by an increase in the product of systolic tension and systolic time, and consequently an increase in oxygen consumption. Our findings are thus in agreement with those of SARNOFF et al. (1958).

Coronary blood flow,

The major part of the coronary flow to the left ventricle takes place during ventricular diastole and it has therefore been assumed that coronary flow would decrease with increased heart rate (GREGG 1950). Such results were reported by ANREP and others (See ANREP, 1926). Opposite results, *i. e.* increased coronary flow with increased rate, were found in our experiments (Figs 1 and 4) and in others (GORLIN 1957, HAUSNER et al. 1940, ESSEX et al. 1943, LAURENT et al. 1956). The changes in coronary vascular tone are very great (Fig. 4).

Altering the heart rate offers a means for studying the regulation of coronary blood flow, for the mechanical conditions (the product of perfusion pressure and perfusion time) and the metabolic factors (oxygen consumption) move in opposite directions when the heart rate is altered. As is seen in Fig. 4, oxygen consumption is a more important determinant of coronary flow than is the coronary perfusion pressure, at least when the latter is held above a certain level. Later studies in this laboratory have demonstrated that this type of regulation may be effective also during such

extreme conditions as cardiac arrest and ventricular fibrillation (BERGLUND, MONROE, and SCHREINER, 1957). The coronary blood flow during these and other conditions (CASE et al., 1955) seems to adjust so that the oxygen tension in the myocardium, and therefore in the capillaries, is maintained at a constant level (BERGLUND, 1955). Even at high heart rates there is no evidence of myocardial hypoxia in the normal heart.

The effect of heart rate on myocardial oxygen consumption and coronary blood flow should be considered when studying the effects of drugs and nervous stimulation on coronary vessels, whether in the intact animal or the isolated heart.

Implications in heart disease.

In the presence of a stenosis in the heart restricting blood flow during diastole, the effects of increased heart rate and the consequent reduction of diastolic time would be more deleterious than during normal conditions. One such circumstance is mitral stenosis in which diastolic filling of the heart takes place at a relatively slow and uniform rate (BRAUNWALD et al. 1955). "If the blood flow to the body is to be maintained despite the increased heart rate, the rate of valvular flow must rise. This can occur only if the pulmonary pressure head increases,... (which) can lead to pulmonary edema" (GORLIN et al. 1951). If the pressure head does not increase the cardiac output will fall.

Another such circumstance is coronary stenosis with its restricting effect on coronary flow. This has been simulated in the experiment illustrated in Fig. 5. With increased rate and reduction of diastolic time the coronary flow apparently could not supply enough oxygen for the increased demand and left ventricular failure developed. The failure was reversed simply by returning the heart rate to a lower level.

Summary.

1. The effects of variations in heart rate on ventricular function, myocardial oxygen consumption, and coronary blood flow were studied in anesthetized, open-chest dogs. The heart rate was held at desired rates by electrical stimulation.

2. The following changes occurred when the rate was increased in steps from 33—80 per min. to 200—280 per min.: cardiac output

increased 0 to 73 per cent and reached a maximum at rates varying from 90 to 180 per min.; above these rates there was usually a decrease. Stroke volume decreased progressively. Mean aortic pressure rose slightly, and the pulse pressure was diminished. Myocardial oxygen consumption and left coronary flow increased, even at the high rates when ventricular work was constant or decreasing; there was a marked fall in coronary resistance.

3. Ventricular function curves, *i. e.* ventricular stroke work plotted against the respective mean atrial pressure, were obtained at the various rates. The curves were always lower at higher than at lower rates.

4. Myocardial oxygen consumption per unit of mechanical work was greater at higher rates, probably because of the larger amount of "isometric work" per min. Coronary blood flow per unit of work increased similarly with rate. Coronary vascular resistance was always much lower at high heart rates than at low, indicating coronary vasodilation.

5. It is concluded that during normal circumstances myocardial oxygen consumption is a major determinant of coronary blood flow.

6. Effects of heart rate during abnormal circulatory conditions were illustrated and discussed.

References.

ANREP, G. V., The regulation of the coronary circulation. Physiol. Rev. 1926. *6.* 596.

ASMUSSEN, E. and M. NIELSEN, Cardiac output during muscular work and its regulation. Physiol. Rev. 1955. *35.* 778.

BARGER, A. C., V. RICHARDS, J. METCALFE, and B. GUNTHER, Regulation of the circulation during exercise. Cardiac output (direct Fick) and metabolic adjustments in the normal dog. Amer. J. Physiol. 1956. *184.* 613.

BERGLUND, E., The function of the ventricles of the heart. Studies on the relation between diastolic filling and ventricular work in the anesthetized dog. Acta physiol. scand. 1955. *33.* Suppl. 119.

BERGLUND, E., R. G. MONROE, and G. L. SCHREINER, Myocardial oxygen consumption and coronary blood flow during cardiac arrest and ventricular fibrillation. Acta physiol. scand. 1957. *41.* 261.

BRAUNWALD, E., H. L. MOSCOVITZ, S. S. AMRAM, R. P. LASSER, S. O. SAPIN, A. HIMMELSTEIN, M. M. RAVITCH, and A. J. GORDON, The hemodynamics of the left side of the heart as studied by simultaneous left atrial, left ventricular, and aortic pressures; particular reference to mitral stenosis. Circulation (N. Y.) 1955. *12*. 69.

BUCKLEY, N. M., E. OGDEN and D. S. LINTON, Jr., The effects of work load and heart rate on filling of the isolated right ventricle of the dog heart. Circulation Res. 1955. *3*. 434.

CASE, R. B., E. BERGLUND and S. J. SARNOFF, Ventricular function. II. Quantitative relationship between coronary flow and ventricular function with observations on unilateral failure. Circulation Res. 1954. *2*. 319.

CASE, R. B., E. BERGLUND and S. J. SARNOFF, Ventricular function. VII. Changes in coronary resistance and ventricular function resulting from acutely induced anemia and the effect thereon of coronary stenosis. Amer. J. Med. 1955. *18*. 317.

COTTON, M. DE V., Circulatory changes affecting measurements of heart force *in situ* with strain gauge arches. Amer. J. Physiol. 1953. *174*. 365.

DUFF, F., E. BERGLUND and H. G. BORST, Effect of heart rate on ventricular function and coronary circulation in dogs. Amer. J. Physiol. 1955. *183*. 611.

ESSEX, H. E., J. F. HERRICK, F. C. MANN and E. J. BALDES, The effect of atropine on the coronary blood flow of trained dogs with denervated and partially denervated hearts. Amer. J. Physiol. 1943. *138*. 683.

EVANS, C. L. and Y. MATSUOKA, The effect of various mechanical conditions on the gaseous metabolism and efficiency of the mammalian heart. J. Physiol. 1914. *49*. 378.

GOLLWITZER-MEIER, K. and E. KRÜGER: Zur Verschiedenheit der Herzenergetik und Herzdynamik bei Druck- und Volumleistung. Pflügers Arch. ges. Physiol. 1936. *238*. 279.

GORLIN, R., Studies on the regulation of the coronary circulation in man. I. Atropine-induced changes in cardiac rate. Am. J. Med. In Press.

GORLIN, R., B. M. LEWIS, F. W. HAYNES, R. J. SPIEGEL and L. DEXTER, Factors regulating pulmonary "capillary" pressure in mitral stenosis. IV. Amer. Heart J. 1951. *41*. 834.

GREGG, D., *Coronary circulation in health and disease.* Lea & Febiger. Philadelphia. 1950.

HAUSNER, E., H. E. ESSEX, J. F. Herrick and E. J. BALDES, Control of coronary blood flow in the heart-lung preparation. Amer. J. Physiol. 1940. *131*. 43.

LAURENT, D., C. BOLENE-WILLIAMS, F. L. WILLIAMS and L. N. KATZ, Effects of heart rate on coronary flow and cardiac oxygen consumption. Amer. J. Physiol. 1956. *185*. 335.

ROHDE, E., Über den Einfluss der mechanischen Bedingungen auf die Tätigkeit und den Sauerstoffverbrauch des Warmblüterherzens. Arch. exp. Path. Pharmak. 1912. *68*. 401.

RUSHMER, R., 1956. Personal communication.

SARNOFF, S. J. and E. BERGLUND, The Potter electroturbinometer. An instrument for recording total systemic blood flow in the dog. Circulation Res. 1953. *1*. 331.

SARNOFF, S. J. and E. BERGLUND, Ventricular function. I. Starling's law of the heart studied by means of simultaneous right and left ventricular function curves in the dog. Circulation (N. Y.) 1954. *9*. 706.

SARNOFF, S. J., E. BRAUNWALD, G. H. WELCH, Jr., R. B. CASE, W. N. STAINSBY and R. MACRUZ, Hemodynamic determinants of the oxygen consumption of the heart, with special reference to the tension-time index. Amer. J. Physiol. In Press.

SHIPLEY, R. E. and C. Wilson, An improved recording rotameter. Proc. Soc. exp. Biol. 1951. *78*. 724.

STARZL, T. E. and R. A. GAERTNER, Chronic heart block in dogs. A method for producing experimental heart failure. Circulation (N. Y.) 1955. *12*. 259.

ULLRICH, K. J., G. RIECKER and K. KRAMER, Das Druckvolumdiagramm des Warmblüterherzens. Isometrische Gleichgewichtskurven. Pflügers Arch. ges. Physiol. 1954. *259*. 481.

ÅSTRAND, P.-O., Experimental studies of physical working capacity in relation to sex and age. Ejnar Munksgaard. Copenhagen. 1952.

NER.

ometer.
he dog.

sarling's
nd left
1954. *9.*

, W. N.
he oxy-
tension-

ameter.

dogs. A
(N. Y.)

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